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(71) Applicant (*for all designated States except US*): COMMONWEALTH SCIENTIFIC AND INDUSTRIAL RESEARCH ORGANISATION [AU/AU]; Limestone Avenue, Campbell, ACT 2601 (AU).

(72) Inventors; and
(75) Inventors/Applicants (*for US only*): JARDINAUD, Marie-Francoise [FR/AU]; 46 Girrahween Street, Braddon, ACT 2612 (AU). DENNIS, Elizabeth, Salisbury [AU/AU]; 100 Hopetoun Circuit, Yarralumla, ACT 2600 (AU). PEACOCK, William, James [AU/AU]; 16 Brassey Street, Deakin, ACT 2600 (AU).

(74) Agents: SLATTERY, John, Michael et al.; Davies Collison Cave, 1 Little Collins Street, Melbourne, VIC 3000 (AU).

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(54) Title: METHOD OF INCREASING THE IRON CONTENT OF PLANT CELLS

(57) Abstract

The present invention provides a method of increasing the bioavailable iron content of a non-animal cell, tissue or organ comprising introducing a genetic sequence which encodes an iron-binding protein, preferably a genetic sequence which encodes a hem-binding protein such as hemoglobin or ferritin, to said non-animal cell, tissue or organ and expressing said genetic sequence therein for a time and under conditions sufficient for the level of said iron-binding protein to be increased. The transformed non-animal cells, tissues and organs produced using the inventive method are of improved nutritive value to animals and humans, particularly in respect of overcoming anaemia and the effects of anaemia. The invention further provides a novel genetic sequence derived from rice which encodes a ferritin polypeptide, amongst others, for use in performing the inventive method.

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METHOD OF INCREASING THE IRON CONTENT OF PLANT CELLS

FIELD OF THE INVENTION

The present invention relates generally to a method of increasing the nutritive value of an organic foodstuff such as a plant, algae, fungus or other foodstuff to a human or non-human animal, by increasing the content of a macronutrient or micronutrient therein using genetic means. In particular, the invention relates to a method of increasing the iron content of a plant, algal, fungal or other non-animal or organism. The present invention is particularly useful for the production of a transgenic non-animal having a high macronutrient or micronutrient content. The invention is also useful in the production of a highly nutritive source of macronutrient or micronutrient and in the production of medicated foodstuffs for increasing the bioavailability of a macronutrient or micronutrient to an animal.

GENERAL

Throughout this specification and the claims which follow, unless the context requires otherwise, the word "comprise", and variations such as "comprises" and "comprising", will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps.

Bibliographic details of the publications referred to by author in this specification are collected at the end of the description. Sequence identity numbers (SEQ ID NOS:) for the nucleotide and amino acid sequences referred to herein are defined after the bibliography.

BACKGROUND TO THE INVENTION

Iron deficiency is the most prevalent nutritional disorder worldwide, the highest prevalence of iron deficiency occurring in Africa and South East Asia. A Swedish study in 1977 reported that 70% of women of childbearing age on a simple Southeast Asian diet consisting of rice, cooked vegetables and spices, could be estimated to maintain their iron balance only in a state of iron deficiency (Haleberg *et al*, 1977).

In a recent report from the CSIRO Division of Human Nutrition, Australia, it has been estimated that 2-4% of Australian women and 3% of healthy children are anaemic. Approximately 4% of all females could be classed as iron deficient, increasing to 10% if they were pregnant or had donated blood in the last 12 months. Individuals from socio-economically disadvantaged groups, ethnic groups (Aborigines, Asian migrants), vegetarians and athletes are also considered to be at particular risk from iron deficiency. Other groups at high risk are adolescents with increased requirements associated with growth and puberty, particularly females with the commencement of menstruation.

Nutritional iron problems associated with vegetarian diets are particularly prevalent in developing countries where major reliance is placed on staple cereals such as rice and wheat, and where intake of animal or fish sources of heme protein are either culturally unacceptable or impractical due to shortage or unreliability of supply, or poverty.

Furthermore, the level of heme iron in vegetarian diets is low and much of the iron in vegetarian diets is poorly absorbed due to the presence of factors such as phytates, condensed tannins, certain non-animal proteins, phosphates, oxalates and phosvitin (egg yolk) that may inhibit iron absorption. In general, iron present in plant material has low bioavailability to humans or other animals because it is poorly absorbed.

The most highly bioavailable (25%) iron comes from meat and fish in which the heme iron forms myoglobin and hemoglobin.

Iron deficiency arises from sustained negative iron balance: the amount of iron absorbed by the body is too low to compensate for the normal physiological requirements. The amount of iron which can be absorbed from the food (bioavailibility) depends on the quantity and chemical form of iron in the diet and the presence of dietary constituents that modify its absorbability (the amount of iron absorbed also depends of the iron status of the individual). Heme iron which is mainly absent from diets composed primarily of vegetable products has a bioavailibility of approximately 25-35% and is not greatly influenced by the

nature of the meal. Non-heme iron has a much greater variability with regard to bioavailibility depending on the nature of the components consumed in a meal.

In adult males, daily iron requirements are of the order of 0.7 - 1 mg (determined by basal losses), in women of fertile age : 1.2 - 1.5 mg, in pregnant women: 4 - 6 mg, and in children of 6 to 12 months, 0.1 mg of Fe/kg of body weight, which almost equals the daily requirements of an adult male.

The consequences of iron deficiency are serious and reflect the degree of deficiency. During pregnancy it is associated with low birth weight, premature delivery, prenatal and fetal death. During childhood, it leads to impaired cognitive performance, motor development and decreased linear growth rate. Adults suffer from reduced ability to do physical work. It also impairs the normal defence systems against infection.

In work leading up to the present invention, the inventors sought to increase the bioavailability to humans and other animals, of iron in non-animal foodstuffs, such as plants, fungi and algae, amongst others, by introducing thereto an iron-binding protein molecule. In particular, the inventors have discovered that the bioavailability of iron may be increased by introducing a genetic sequence which encodes a heme protein into a plant cell and expressing said genetic sequence therein.

SUMMARY OF THE INVENTION

One aspect of the invention provides a method of increasing the bioavailable iron content of a non-animal organism, organ, tissue or cell, said method comprising the steps of introducing thereto a genetic sequence which encodes an iron-binding protein and expressing said protein therein for a time and under conditions sufficient for the level of said iron-binding protein to increase.

A further aspect of the invention provides a non-animal organism produced using the subject method wherein said non-animal organism or a cell, tissue or organ thereof comprises

a higher level of bioavailable iron in a protein-bound form than an otherwise isogenic organism which does not contain the introduced genetic sequence encoding the iron-binding protein.

A further aspect of the invention provides a plant produced using the subject method wherein the seed of said plant comprises a higher level of bioavailable iron in a protein-bound form than an otherwise isogenic plant which does not contain the introduced genetic sequence encoding the iron-binding protein.

A further aspect of the invention provides an isolated nucleic acid molecule which comprises a nucleotide sequence which is at least about 85% identical to the nucleotide sequence set forth in SEQ ID NO:1 or a complementary nucleotide sequence thereto when used to increase the bioavailable iron content in a plant.

A further aspect of the invention provides an isolated nucleic acid molecule which comprises a nucleotide sequence which is at least about 85% identical to the nucleotide sequence set forth in SEQ ID NO:3 or a complementary nucleotide sequence thereto or a homologue, analogue or derivative of said nucleotide sequence which is capable of encoding an iron-binding peptide, polypeptide or protein.

A further aspect of the invention provides an isolated nucleic acid molecule which comprises a nucleotide sequence which is at least about 85% identical to the nucleotide sequence set forth in SEQ ID NO:5 or a complementary nucleotide sequence thereto when used to increase the bioavailable iron content in a plant.

A further aspect of the invention provides an isolated nucleic acid molecule which comprises a nucleotide sequence which encodes a peptide, polypeptide or protein having at least about 85% amino acid sequence similarity to the sequence set forth in SEQ ID NO:2 when used to increase the bioavailable iron content in a plant.

A further aspect of the invention provides an isolated nucleic acid molecule which

comprises a nucleotide sequence which encodes an iron-binding peptide, polypeptide or protein having at least about 85% amino acid sequence similarity to the sequence set forth in SEQ ID NO:4.

A further aspect of the invention provides an isolated nucleic acid molecule which comprises a nucleotide sequence which encodes a peptide, polypeptide or protein having at least about 85% amino acid sequence similarity to the sequence set forth in SEQ ID NO:6 when used to increase the bioavailable iron content in a plant.

A further aspect of the invention provides a cell which has been transformed or transfected with the isolated nucleic acid molecule.

A further aspect of the invention provides a plant tissue other than a whole plant regenerated from the cell.

A further aspect of the invention provides a method of treatment of iron deficiency in a human or animal subject comprising administering to said subject plant tissue or a derivative thereof having a high bioavailable iron content for a time and under conditions sufficient for the level of iron detectable in the blood of said subject to increase, wherein said plant tissue has a high bioavailable iron content by virtue of the expression therein of an introduced genetic sequence which encodes and iron-binding peptide, polypeptide or protein.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a diagrammatic representation of the plasmid pActl-HbAra-tNOS, which comprises the *Arabidopsis thaliana* hemoglobin gene (HbAra) placed operably under the control of the actin promoter (pAct1) and upstream of the nopaline synthase terminator sequence (tNOS).

Figure 2 is a diagrammatic representation of the plasmid pGt1fl-HbRice-3'Bt2, which comprises the rice hemoglobin gene (HbRice) placed operably under the control of the rice

glutelin promoter (Gt1fl) and upstream of the rice ADP-glucose pyrophosphorylase terminator sequence (t3'Bt2).

Figure 3 is a diagrammatic representation of the plasmid pGt1fl-HbAra-3'Bt2, which comprises the *Arabidopsis thaliana* hemoglobin gene (HbAra) placed operably under the control of the rice glutelin promoter (Gt1fl) and upstream of the rice ADP-glucose pyrophosphorylase terminator sequence (t3'Bt2).

Figure 4 is a diagrammatic representation of the plasmid pActl-HbRice-tNOS, which comprises the rice hemoglobin gene (HbRice) placed operably under the control of the actin promoter (pAct1) and upstream of the nopaline synthase terminator sequence (tNOS).

Figure 5 is a diagrammatic representation of the plasmid pHMW-HbAra-tNOS, which comprises the *Arabidopsis thaliana* hemoglobin gene (HbAra) placed operably under the control of the HMW promoter (pHMW) and upstream of the nopaline synthase terminator sequence (tNOS).

Figure 6 is a diagrammatic representation of the plasmid pHMW-HbRice-tNOS, which comprises the rice hemoglobin gene (HbRice) placed operably under the control of the HMW promoter (pHMW) and upstream of the nopaline synthase terminator sequence (tNOS).

Figure 7 is a diagrammatic representation of the plasmid pGT1-sh1HbRice-tNOS, which comprises the rice hemoglobin gene (HbRice) placed operably under the control of the rice glutelin promoter (pGt1) and downstream of the *shrunken*1 gene intron 1 sequence (*Sh1* intron) and upstream of the *Agrobacterium tumefaciens* nopaline synthase terminator sequence (tNOS).

Figure 8 is a diagrammatic representation of the plasmid pGT1-sh1HbAra-tNOS, which comprises the *Arabidopsis thaliana* hemoglobin gene (HbAra) placed operably under the control of the rice glutelin promoter (pGt1) and downstream of the *shrunken*1 gene intron 1

sequence (*Sh1* intron) and upstream of the *Agrobacterium tumefaciens* nopaline synthase terminator sequence (tNOS).

Figure 9 is a schematic representation showing the construction of the expression plasmid pActl-HbAra-tNOS.

Figure 10 is a schematic representation showing the construction of the expression plasmid pGt1fl-HbRice-3'Bt2.

Figure 11 is a schematic representation showing the construction of the expression plasmid pGt1fl-HbAra-3'Bt2.

Figure 12 is a schematic representation showing the construction of the expression plasmid pHMW-HbAra-tNOS.

Figure 13 is a schematic representation showing the construction of the expression plasmid pHMW-HbRice-tNOS.

Figure 14 is a schematic representation showing the construction of the expression plasmid pGT1-sh1HbRice-tNOS.

Figure 15 is a schematic representation showing the construction of the expression plasmid pGT1-sh1HbAra-tNOS.

Figure 16 is a diagrammatic representation of the plasmid pGT1-sh1-OSfer-tNOS, which comprises the rice ferritin cDNA (OSfer) placed operably under the control of the rice glutelin promoter (pGt1) and downstream of the *shrunken*1 gene intron 1 sequence (*Sh1* intron) and upstream of the *Agrobacterium tumefaciens* nopaline synthase terminator sequence (tNOS).

Figure 17 is a diagrammatic representation of the plasmid pGt1fl-OSfer-3'Bt2, which comprises the rice ferritin cDNA (OSfer) placed operably under the control of the rice glutelin promoter (Gt1fl) and upstream of the rice ADP-glucose pyrophosphorylase terminator sequence (t3'Bt2).

Figure 18 is a diagrammatic representation of the plasmid pWUbi-OSferMito, which comprises the rice ferritin cDNA (OSfer) placed operably under the control of the ubiquitin gene promoter (Ubi) and upstream of the tm1 sequence.

Figure 19 is a diagrammatic representation of the plasmid pHMW-OSfer-tNOS, which comprises the rice ferritin cDNA (OSfer) placed operably under the control of the HMW promoter (pHMW) and upstream of the nopaline synthase terminator sequence (tNOS).

Figure 20 is a diagrammatic representation of the plasmid pBx17-OSfer-tNOS, which comprises the rice ferritin cDNA (OSfer) placed operably under the control of the full-length HMW promoter (pBx17) and upstream of the nopaline synthase terminator sequence (tNOS).

Figure 21 is a copy of a photographic representation showing copy number and locus number of the rice hemoglobin cDNA expressed under control of the truncated glutelin promoter (HMW-HbR) in transgenic T_0 rice plants. Lanes marked HMW-HbR (i.e. lanes 3-9 from left in the two left panels and lanes 2-8 in the right panel) contained DNA from transformed rice lines. Lanes marked C contained DNA from an untransformed rice line.

Figure 22 is a copy of a Southern blot hybridisation showing hybridisation to the rice hemoglobin cDNA in T_1 rice plants following transformation with a genetic construct containing the rice hemoglobin cDNA operably under the control of the HMW promoter sequence (i.e. HMW-HbR). The lane marked C contains DNA from an untransformed control rice plant. At the bottom of the panel, + indicates the presence of the introduced hemoglobin cDNA; the minus sign (-) indicates the absence of the introduced hemoglobin cDNA from the T_1 line.

Figure 23 is a copy of a western blot showing the presence of recombinant hemoglobin in plants transformed with the rice hemoglobin cDNA placed operably under the control of the rice Actin1 gene promoter. The arrow indicates the position of the hemoglobin polypeptide. The lane marked C contains DNA from an untransformed control rice plant.

Figure 24 is a copy of a western blot showing the presence of recombinant hemoglobin in T2 seed samples of several rice plants transformed with the rice hemoglobin cDNA. In homozygous lines, all seed tested expressed the recombinant hemoglobin polypeptide. In heterozygous lines, recombinant hemoglobin was not detectable in all seed tested. The arrow indicates the position of the hemoglobin polypeptide. The lane marked C contains DNA from an untransformed control rice plant.

Figure 25 is a copy of a western blot showing the signal obtained for 5ng, 50ng and 100 ng of purified hemoglobin polypeptide (left panel) compared to the hemoglobin-specific signal obtained for 10-100 μg of soluble seed protein derived from the seeds of homozygous lines expressing recombinant rice hemoglobin under the control of the GT1 promoter sequence (right panel).

Figure 26 is a copy of a western blot (top) and graphical representation (bottom) showing the level of recombinant hemoglobin contained in seed protein extracts derived from homozygous T1 rice lines expressing hemoglobin cDNAs operably under the control of the presence of recombinant hemoglobin in plants transformed with the rice hemoglobin cDNA placed operably under the control of the HMW promoter (HMW-HbR), or the *Arabidopsis thaliana* hemoglobin cDNA placed operably under the control of either the rice Actin1 gene promoter (Act-HbA) or the HMW promoter (HMW-HbA) or the GT1 promoter (GT1-HbA). Taipei is an untransformed control rice plant line. Units of expression are indicated relative to the expression obtained using the HMW promoter to drive expression of the *Arabidopsis thaliana* hemoglobin cDNA.

Figure 27 is a representation of an amino acid sequence alignment between maize ferritin

(FM1) and rice ferritin (OSfer) polypeptides. The amino acid sequence of the maize ferritin transit peptide is underlined. The asterisk indicates the cleavage site between the mature subunit and the transit peptide.

Figure 28 is a copy of a photographic representation of a western blot showing expression of rice ferritin (OSFer) in two untransformed rice lines (lanes marked Controls- lanes 2-3) and nine transgenic rice lines transformed with the short rice ferritin cDNA (OSFer short) placed under control of the ubiquitin promoter sequence (lanes 4-12). The position of purified recombinant ferritin is indicated in the first lane. For each sample, 50 μg plant protein or 1 μg purified recombinant ferritin was loaded onto the gel. Antibodies were used at a dilution of 1:4000.

Figure 29 is a copy of a photographic representation of a western blot showing expression of rice ferritin (OSFer) in three untransformed rice lines (lanes marked Controls- lanes 2-4) and eight transgenic rice lines transformed with the long rice ferritin cDNA (OSFer long) placed under control of the ubiquitin promoter sequence (lanes 5-12). The position of purified recombinant ferritin is indicated in the first lane. For each sample, 50 μg plant protein or 1 μg purified recombinant ferritin was loaded onto the gel. Antibodies were used at a dilution of 1:4000.

Figure 30 is a copy of a photographic representation of a western blot showing expression of rice ferritin (OSFer) in different tissues of transgenic rice lines transformed with the long rice ferritin cDNA (OSFer long) placed under control of the ubiquitin promoter sequence (lanes 5-12). The position of purified recombinant ferritin is indicated in the first lane. For each sample, 50 μg plant protein or 1 μg purified recombinant ferritin was loaded onto the gel. Antibodies were used at a dilution of 1:4000.

Figure 31 is a copy of a photographic representation of a western blot showing expression of rice ferritin (OSFer) in the seeds of transgenic rice lines transformed with the rice ferritin

cDNA (OSFer) and in the seeds of untransformed plants (control). The position of purified recombinant ferritin is indicated. For each sample, 50 μg plant protein or 1 μg purified recombinant ferritin was loaded onto the gel. Antibodies were used at a dilution of 1:4000.

Figure 32 is a copy of a photographic representation showing the expression of recombinant ferritin produced in *Escherichia coli* and purified recombinant rice ferritin.

DETAILED DESCRIPTION OF THE INVENTION

Accordingly, one aspect of the invention provides a method of increasing the bioavailable iron-content of a non-animal organism such as a plant, fungus, bacterium or algae, amongst others, said method comprising introducing to said organism a a genetic sequence which is capable of expressing an iron-binding protein and expressing said protein therein.

As used herein, the term "bioavailable" shall be taken to refer to the availability of a stated integer or group of integers to human or animal organism, including the amount of a stated integer or group of integers which is present in a non-animal organism or foodstuff and which is absorbed in the digestive system or blood of a human or other animal following administration thereto or medicated foodstuff which contains the integer or group of integers.

As used herein, the term "bioavailable iron" or "bioavailable iron-content" or similar term shall be taken to refer to the amount of iron, in the ferrous or ferric form, which is present in a non-animal organism or foodstuff and which is absorbed in the digestive system or blood of a human or other animal following administration thereto.

The term "non-animal" refers to any organism other than a mammal, bird, amphibian, reptile, insect or other animal, or a tissue or cell desirable or derived from said organism. In the present context, the term "non-animal" includes plants, fungi, bacteria and algae.

The term "non-animal foodstuff" shall be taken to refer to any foodstuff, for nutritional or medical purposes, in liquid or solid form, which is derived from a non-animal source. Accordingly, a non-animal foodstuff includes any solid or liquid vegetable matter or a tonic, elixir, capsule, tablet, powder or solution comprising same, whether suitable for administration to a human or other animal in a ingestible or injectable format. Furthermore, a non-animal foodstuff may be highly-processed such that it is suitable for administration to a human or other animal by injection or ingestion.

The present invention encompasses the use of any and all such non-animal foodstuffs.

As used herein, the term "derived from" shall be taken to indicate that a particular integer or group of integers has originated from the species specified, but has not necessarily been obtained directly from the specified source.

In a preferred embodiment of the present invention, the non-animal organism is a plant, in particular an edible plant such as, but not limited to, a cereal, selected from the list comprising rice, wheat, maize, sorghum, rye, barley and millet, or a non-cereal selected from the list comprising legumes, vegetable crop plants and tuber-bearing plants.

In a more particularly preferred embodiment of the invention, the plant is a rice plant, even more particularly an indica rice plant such as cultivar selected from the list comprising Arlesienne and Doongarra, amongst others or a japonica rice plant such as cultivar selected from the list comprising Calrose, Millin, Nippon barre, YRM 43 and Jarrah amongst others.

The present invention is clearly applicable to altering the bioavailable iron content of any tissue, cell or organ-types derived from a plant according to the embodiments described herein, for example leaf, fruit, seed, root or tuber material, amongst others.

The term "iron-binding protein" as used herein refers to any polypeptide, peptide or a homologue, analogue or derivative thereof which is at least capable of binding iron as Fe^{2+}

or Fe^{3+}, either as free Fe^{2+} or Fe^{3+} alternatively, in the porphyrin form, for example as iron-porphyrin, iron-protoporphyrin, heme-iron or iron associated with a chlorophyll, cytochrome, cytochrome P_{450} or other porphyrin molecule.

In a preferred embodiment of the invention, the iron-binding protein is a heme protein, more preferably a hemoglobin, myoglobin or ferritin polypeptide or a homologue, analogue or derivative thereof. Wherein the iron-binding protein is a hemoglobin, it is particularly preferred that the hemoglobin is derived from a plant tissue, still more preferably from a plant tissue other than a nitrogen-fixing nodule.

Preferably, the iron-binding protein is expressed in the plant tissue such that it accumulates in the vacuoles or the apoplastic space.

More preferably, the iron-binding protein comprises a sequence of amino acids set forth in SEQ ID NOS:2 or 4 or 6 or a homologue, analogue or derivative thereof which possesses iron-binding activity.

In the present context, "homologues" of an iron-binding protein or an iron-binding polypeptide refer to those polypeptides, enzymes or proteins which have a similar iron-binding activity as the iron-binding protein described herein, notwithstanding any amino acid substitutions, additions or deletions thereto. A homologue may be isolated or derived from the same or another species.

Furthermore, the amino acids of a homologous polypeptide may be replaced by other amino acids having similar properties, for example hydrophobicity, hydrophilicity, hydrophobic moment, charge or antigenicity, and so on.

"Analogues" encompass iron-binding proteins and polypeptides as hereinbefore defined, notwithstanding the occurrence of any non-naturally occurring amino acid analogues therein.

The term "derivative" in relation to an iron-binding protein or iron-binding polypeptide as hereinbefore defined shall be taken to refer hereinafter to mutants, parts or fragments of an iron-binding protein or polypeptide to which ligands are attached to one or more of the amino acid residues contained therein, such as carbohydrates, enzymes, proteins, polypeptides or reporter molecules such as radionuclides or fluorescent compounds. Glycosylated, fluorescent, acylated or alkylated forms of the subject peptides are particularly contemplated by the present invention. Additionally, derivatives of a repressor polypeptide as hereinbefore defined may comprise fragments or parts of an amino acid sequence disclosed herein and are within the scope of the invention, as are homopolymers or heteropolymers comprising two or more copies of the subject polypeptides. Procedures for derivatizing peptides are well-known in the art.

In the context of the present invention, a homologue, analogue or derivative of an iron-binding protein or iron-binding polypeptide will possess iron-binding capacity, however it may not perform the same enzymatic function as the iron-binding protein from which it is derived, or to which it is closely related.

Substitutions encompass amino acid alterations in which an amino acid is replaced with a different naturally-occurring or a non-conventional amino acid residue. Such substitutions may be classified as "conservative", in which case an amino acid residue contained in a repressor polypeptide is replaced with another naturally-occurring amino acid of similar character, for example Gly↔Ala, Val↔Ile↔Leu, Asp↔Glu, Lys↔Arg, Asn↔Gln or Phe↔Trp↔Tyr.

Substitutions encompassed by the present invention may also be "non-conservative", in which an amino acid residue which is present in an iron-binding polypeptide is substituted with an amino acid having different properties, such as a naturally-occurring amino acid from a different group (eg. substituted a charged or hydrophobic amino acid with alanine), or alternatively, in which a naturally-occurring amino acid is substituted with a non-conventional amino acid.

Amino acid substitutions are typically of single residues, but may be of multiple residues, either clustered or dispersed.

Naturally-occurring amino acids include those listed in Table 1. Non-conventional amino acids encompassed by the invention include, but are not limited to those listed in Table 2.

Amino acid deletions will usually be of the order of about 1-10 amino acid residues, while insertions may be of any length. Deletions and insertions may be made to the N-terminus, the C-terminus or be internal deletions or insertions. Generally, insertions within the amino acid sequence will be smaller than amino-or carboxyl-terminal fusions and of the order of 1-4 amino acid residues.

Those skilled in the art will be aware of the means of producing analogues and derivatives of an iron-binding protein. In one approach, analogues are produced by genetic means wherein genetic sequences which encode the iron-binding protein are mutagenised, such that the resultant polypeptide encoded thereby comprises an altered amino acid sequence. Such methods are well within the means of the skilled artisan.

In one particularly preferred embodiment of the invention, the iron binding protein is a plant-derived hemoglobin. Plant hemoglobins are effective in the performance of the invention by virtue of the fact that each hemoglobin polypeptide is able to bind a single atom of iron. According to this embodiment, the genetic sequences expressing plant hemoglobin may be derived from any plant organ including the nodules, roots and leaves amongst others. Furthermore, the genetic sequence may be derived from any plant species. In a particularly preferred embodiment however, the species of plant from which the genetic sequence is derived is selected from the list comprising *Arabidopsis thaliana*, *Oryza sativa* and maize.

TABLE 1

Amino Acid	Three-letter Abbreviation	One-letter Symbol
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V
Any amino acid as above	Xaa	X

TABLE 2

Non-conventional amino acid	Code	Non-conventional amino acid	Code
α-aminobutyric acid	Abu	L-N-methylalanine	Nmala
α-amino-α-methylbutyrate	Mgabu	L-N-methylarginine	Nmarg
aminocyclopropane-carboxylate	Cpro	L-N-methylasparagine	Nmasn
		L-N-methylaspartic acid	Nmasp
aminoisobutyric acid	Aib	L-N-methylcysteine	Nmcys
aminonorbornyl-carboxylate	Norb	L-N-methylglutamine	Nmgln
		L-N-methylglutamic acid	Nmglu
cyclohexylalanine	Chexa	L-N-methylhistidine	Nmhis
cyclopentylalanine	Cpen	L-N-methylisolleucine	Nmile
D-alanine	Dal	L-N-methylleucine	Nmleu
D-arginine	Darg	L-N-methyllysine	Nmlys
D-aspartic acid	Dasp	L-N-methylmethionine	Nmmet
D-cysteine	Dcys	L-N-methylnorleucine	Nmnle
D-glutamine	Dgln	L-N-methylnorvaline	Nmnva
D-glutamic acid	Dglu	L-N-methylornithine	Nmorn
D-histidine	Dhis	L-N-methylphenylalanine	Nmphe
D-isoleucine	Dile	L-N-methylproline	Nmpro
D-leucine	Dleu	L-N-methylserine	Nmser
D-lysine	Dlys	L-N-methylthreonine	Nmthr
D-methionine	Dmet	L-N-methyltryptophan	Nmtrp
D-ornithine	Dorn	L-N-methyl tyrosine	Nmtyr
D-phenylalanine	Dphe	L-N-methylvaline	Nmval
D-proline	Dpro	L-N-methylethylglycine	Nmetg
D-serine	Dser	L-N-methyl-t-butylglycine	Nmtbug
D-threonine	Dthr	L-norleucine	Nle

D-tryptophan	Dtrp
D-tyrosine	Dtyr
D-valine	Dval
D-α-methylalanine	Dmala
D-α-methylarginine	Dmarg
D-α-methylasparagine	Dmasn
D-α-methylaspartate	Dmasp
D-α-methylcysteine	Dmcys
D-α-methylglutamine	Dmgln
D-α-methylhistidine	Dmhis
D-α-methylisoleucine	Dmile
D-α-methylleucine	Dmleu
D-α-methyllysine	Dmlys
D-α-methylmethionine	Dmmet
D-α-methylornithine	Dmorn
D-α-methylphenylalanine	Dmphe
D-α-methylproline	Dmpro
D-α-methylserine	Dmser
D-α-methylthreonine	Dmthr
D-α-methyltryptophan	Dmtrp
D-α-methyltyrosine	Dmty
D-α-methylvaline	Dmval
D-N-methylalanine	Dnmala
D-N-methylarginine	Dnmarg
D-N-methylasparagine	Dnmasn
D-N-methylaspartate	Dnmasp
D-N-methylcysteine	Dnmcys

L-norvaline	Nva
α-methyl-aminoisobutyrate	Maib
α-methyl-γ-aminobutyrate	Mgabu
α-methylcyclohexylalanine	Mchexa
α-methylcylcopentylalanine	Mcpen
α-methyl-α-napthylalanine	Manap
α-methylpenicillamine	Mpen
N-(4-aminobutyl)glycine	Nglu
N-(2-aminoethyl)glycine	Naeg
N-(3-aminopropyl)glycine	Norn
N-amino-α-methylbutyrate	Nmaabu
α-napthylalanine	Anap
N-benzylglycine	Nphe
N-(2-carbamylethyl)glycine	Ngln
N-(carbamylmethyl)glycine	Nasn
N-(2-carboxyethyl)glycine	Nglu
N-(carboxymethyl)glycine	Nasp
N-cyclobutylglycine	Ncbut
N-cycloheptylglycine	Nchep
N-cyclohexylglycine	Nchex
N-cyclodecylglycine	Ncdec
N-cylcododecylglycine	Ncdod
N-cyclooctylglycine	Ncoct
N-cyclopropylglycine	Ncpro
N-cycloundecylglycine	Ncund
N-(2,2-diphenylethyl) glycine	Nbhm
N-(3,3-diphenylpropyl) glycine	Nbhe

D-N-methylglutamine	Dnmgln	N-(3-guanidinopropyl) glycine	Narg
D-N-methylglutamate	Dnmglu	N-(1-hydroxyethyl)glycine	Nthr
D-N-methylhistidine	Dnmhis	N-(hydroxyethyl))glycine	Nser
D-N-methylisoleucine	Dnmile	N-(imidazolylethyl)) glycine	Nhis
D-N-methylleucine	Dnmleu	N-(3-indolylyethyl) glycine	Nhtrp
D-N-methyllysine	Dnmlys	N-methyl-γ-aminobutyrate	Nmgabu
N-methylcyclohexylalanine	Nmchexa	D-N-methylmethionine	Dnmmet
D-N-methylornithine	Dnmorn	N-methylcyclopentylalanine	Nmcpen
N-methylglycine	Nala	D-N-methylphenylalanine	Dnmphe
N-methylaminoisobutyrate	Nmaib	D-N-methylproline	Dnmpro
N-(1-methylpropyl)glycine	Nile	D-N-methylserine	Dnmser
N-(2-methylpropyl)glycine	Nleu	D-N-methylthreonine	Dnmthr
D-N-methyltryptophan	Dnmtrp	N-(1-methylethyl)glycine	Nval
D-N-methyltyrosine	Dnmtyr	N-methyla-napthylalanine	Nmanap
D-N-methylvaline	Dnmval	N-methylpenicillamine	Nmpen
γ-aminobutyric acid	Gabu	N-(*p*-hydroxyphenyl)glycine	Nhtyr
L-*t*-butylglycine	Tbug	N-(thiomethyl)glycine	Ncys
L-ethylglycine	Etg	penicillamine	Pen
L-homophenylalanine	Hphe	L-α-methylalanine	Mala
L-α-methylarginine	Marg	L-α-methylasparagine	Masn
L-α-methylaspartate	Masp	L-α-methyl-*t*-butylglycine	Mtbug
L-α-methylcysteine	Mcys	L-methylethylglycine	Metg
L-α-methylglutamine	Mgln	L-α-methylglutamate	Mglu
L-α-methylhistidine	Mhis	L-α-methylhomo phenylalanine	Mhphe
L-α-methylisoleucine	Mile	N-(2-methylthioethyl) glycine	Nmet

L-α-methylleucine	Mleu	L-α-methyllysine	Mlys
L-α-methylmethionine	Mmet	L-α-methylnorleucine	Mnle
L-α-methylnorvaline	Mnva	L-α-methylornithine	Morn
L-α-methylphenylalanine	Mphe	L-α-methylproline	Mpro
L-α-methylserine	Mser	L-α-methylthreonine	Mthr
L-α-methyltryptophan	Mtrp	L-α-methyltyrosine	Mtyr
L-α-methylvaline	Mval	L-N-methylhomo phenylalanine	Nmhphe
N-(N-(2,2-diphenylethyl) carbamylmethyl)glycine	Nnbhm	N-(N-(3,3-diphenylpropyl) carbamylmethyl)glycine	Nnbhe
1-carboxy-1-(2,2-diphenyl-ethylamino)cyclopropane	Nmbc		

The present invention extends to the use of a genetic sequence encoding a plant hemoglobin which comprises a sequence of nucleotides or is complementary to a sequence of nucleotides substantially as set forth in SEQ ID NO:1 or at least 70% identical thereto. Preferably, the percentage identity to SEQ ID NO:1 or a complementary sequence thereto is at least 80%, more preferably at least 90% and still more preferably at least about 99%.

In an alternative embodiment, the plant-derived hemoglobin or homologue, analogue or derivative thereof comprises a sequence of amino acids substantially as set forth in SEQ ID NO:2 or at least 70%, preferably at least 80%, more preferably at least 90% and even more preferably at least about 99% similar to SEQ ID NO:2.

For the purposes of nomenclature, the nucleotide sequence set forth in SEQ ID NO:1 relates to the *Arabidopsis thaliana* hemoglobin genomic gene. The present invention clearly extends to genetic constructs which comprise the complement of SEQ ID NO:1 or homologues, analogues or derivatives thereof which may be readily derivable by those skilled in the art. The amino acid sequence set forth in SEQ ID NO:2 relates to *Arabidopsis thaliana*

hemoglobin, encoded by the gene set forth in SEQ ID NO:1 or an equivalent nucleotide sequence, such as a cDNA or RNA molecule, or synthetic DNA molecule.

In an alternative embodiment which is particularly preferred in performing the invention, the iron-binding protein is a ferritin peptide, polypeptide or protein, preferably a plant-derived ferritin peptide, polypeptide or protein or a homologue, analogue or derivative thereof.

According to this embodiment, it is particularly preferred that the genetic sequences expressing ferritin may be derived from a maize or a rice plant. Other sources are not excluded.

More preferably, the ferritin genetic sequence or homologue, analogue or derivative thereof comprises a sequence of nucleotides which is at least 85% identical to the nucleotide sequence set forth in SEQ ID NOS:3 or 5 or a complementary nucleotide sequence thereof. Even more preferably, the percentage identity to SEQ ID NOS:3 or 5 is at least 90%, still more preferably at least 95% and even still more preferably at least 99% or 100% identical to SEQ ID NOS:3 or 5.

In an alternative embodiment, the plant-derived ferritin peptide, polypeptide or protein or a homologue, analogue or derivative thereof comprises a sequence of amino acids substantially as set forth in SEQ ID NOS:4 or 6 or is at least 85%, preferably at least 90%, more preferably at least 95% and even more preferably at least about 99% similar to SEQ ID NOS:4 or 6.

For the purposes of nomenclature, the nucleotide sequence set forth in SEQ ID NO:3 relates to the *Oryza sativa* ferritin cDNA sequence. The nucleotide sequence set forth in SEQ ID NO:5 relates to the ferritin cDNA sequence. The amino acid sequences set forth in SEQ ID NO:4 and SEQ ID NO:6 relate to the *Oryza sativa* and *Zea mays* ferritin polypeptides, respectively encoded by said cDNA sequences.

In determining whether or not two nucleotide sequences fall within these percentage limits, those skilled in the art will be aware that it is necessary to conduct a side-by-side comparison of nucleotide sequences. In such comparisons or alignments, differences will arise in the positioning of non-identical nucleotide residues, depending upon the algorithm used to perform the alignment. Similar considerations arise in the comparison of two or more amino acid sequences. In the present context, reference to a percentage similarity between two or more nucleotide sequences or amino acid sequences shall be taken to refer to the number of identical and similar residues between said sequences as determined using any standard algorithm known to those skilled in the art. For example, nucleotide sequence and amino acid sequence identities or similarities may be calculated using the BESTFIT and GAP programmes, respectively, of the Computer Genetics Group, Inc., University Research Park, Madison, Wisconsin, United States of America (Devereaux *et al*, 1984). The GAP programme utilizes the algorithm of Needleman and Wunsch (1970) to maximise the number of identical/similar residues and to minimise the number and/or length of sequence gaps in the alignment. Alternatively, wherein two amino acid sequences are being compared, the ClustalW programme of Thompson *et al*. (1994) is used.

The genetic sequence which encodes an iron-binding protein as defined herein, may be derived from a classical genomic gene, comprising introns and exons or alternatively, the subject genetic sequence may be a cDNA molecule, Expressed Sequence Tag (EST), RNA molecule or a synthetic oligonucleotide molecule, the only requirement being that said genetic sequence, when expressed, is capable of encoding a polypeptide which is capable of binding iron.

The present invention clearly extends to the use of homologues, analogues and derivatives of a hemoglobin or ferritin nucleotide sequence which is at least capable of encoding a polypeptide which binds iron in such a way as to increase the available iron content of a plant cell in which said polypeptide is expressed. The present invention extends further to the use of publicly available genetic sequences not specifically disclosed herein, the only requirement being that such genetic sequences are capable of encoding a polypeptide

which binds iron.

For the present purpose, "homologues" of a nucleotide sequence shall be taken to refer to an isolated nucleic acid molecule which is substantially the same as the nucleic acid molecule of the present invention or its complementary nucleotide sequence, notwithstanding the occurrence within said sequence, of one or more nucleotide substitutions, insertions, deletions, or rearrangements.

"Analogues" of a nucleotide sequence set forth herein shall be taken to refer to an isolated nucleic acid molecule which is substantially the same as a nucleic acid molecule of the present invention or its complementary nucleotide sequence, notwithstanding the occurrence of any non-nucleotide constituents not normally present in said isolated nucleic acid molecule, for example carbohydrates, radiochemicals including radionucleotides, reporter molecules such as, but not limited to DIG, alkaline phosphatase or horseradish peroxidase, amongst others.

"Derivatives" of a nucleotide sequence set forth herein shall be taken to refer to any isolated nucleic acid molecule which contains significant sequence similarity to said sequence or a part thereof. Generally, the nucleotide sequence of the present invention may be subjected to mutagenesis to produce single or multiple nucleotide substitutions, deletions and/or insertions. Nucleotide insertional derivatives of the nucleotide sequence of the present invention include 5´ and 3´ terminal fusions as well as intra-sequence insertions of single or multiple nucleotides or nucleotide analogues. Insertional nucleotide sequence variants are those in which one or more nucleotides or nucleotide analogues are introduced into a predetermined site in the nucleotide sequence of said sequence, although random insertion is also possible with suitable screening of the resulting product being performed. Deletional variants are characterised by the removal of one or more nucleotides from the nucleotide sequence. Substitutional nucleotide variants are those in which at least one nucleotide in the sequence has been removed and a different nucleotide or nucleotide analogue inserted in its place.

Homologues, analogues and derivatives of the nucleotide sequences set forth in SEQ ID NOS:1, 3 and 5 may be obtained by any standard procedure known to those skilled in the art, such as by nucleic acid hybridization (Ausubel *et al*, 1987), polymerase chain reaction (McPherson *et al*, 1991) screening of expression libraries using antibody probes (Huynh *et al*, 1983) or by functional assay.

In nucleic acid hybridizations, genomic DNA, mRNA or cDNA or a part of fragment thereof, in isolated form or contained within a suitable cloning vector such as a plasmid or bacteriophage or cosmid molecule, is contacted with a hybridization-effective amount of a nucleic acid probe derived from SEQ ID NOS:1 or 3 or 5 for a time and under conditions sufficient for hybridization to occur and the hybridized nucleic acid is then detected using a detecting means.

Detection is performed preferably by labelling the probe with a reporter molecule capable of producing an identifiable signal, prior to hybridization. Preferred reporter molecules include radioactively-labelled nucleotide triphosphates and biotinylated molecules.

For the purposes of defining the level of stringency, a low stringency is defined herein as being a hybridisation and/or a wash carried out in 6xSSC buffer, 0.1 % (w/v) SDS at 28°C. Generally, the stringency is increased by reducing the concentration of SSC buffer, and/or increasing the concentration of SDS and/or increasing the temperature of the hybridisation and/or wash. Conditions for hybridisations and washes are well understood by one normally skilled in the art. For the purposes of clarification of parameters affecting hybridisation between nucleic acid molecules, reference can conveniently be made to pages 2.10.8 to 2.10.16. of Ausubel *et al.* (1987), which is herein incorporated by reference.

Preferably, variants of the hemoglobin or ferritin gene exemplified herein are isolated by hybridisation under medium or more preferably, under high stringency conditions, to a probe which comprises at least about 30 contiguous nucleotides derived from SEQ ID NOS:

1 or 3 or 5 or a complement thereof.

In the polymerase chain reaction (PCR), a nucleic acid primer molecule comprising at least about 14 nucleotides in length derived from SEQ ID NOS:1 or 3 or 5 or a complementary sequence thereto is hybridized to a nucleic acid template molecule and specific nucleic acid molecule copies of the template are amplified enzymatically as described in McPherson *et al*, (1991), which is incorporated herein by reference.

In expression screening of cDNA libraries or genomic libraries, protein- or peptide-encoding regions are placed operably under the control of a suitable promoter sequence in the sense orientation, expressed in a prokaryotic cell or eukaryotic cell in which said promoter is operable to produce a peptide or polypeptide, screened with a monoclonal or polyclonal antibody molecule or a derivative thereof against one or more epitopes of a hemoglobin or ferritin polypeptide and the bound antibody is then detected using a detecting means, essentially as described by Huynh *et al* (1985) which is incorporated herein by reference. Suitable detecting means according to this embodiment include ^{125}I-labelled antibodies or enzyme-labelled antibodies capable of binding to the first-mentioned antibody, amongst others.

The present invention clearly extends to any one of the nucleotide sequences set forth in SEQ ID NOS:1 or 3 or 5 when used in the inventive method described herein, to increase the bioavailable iron content of a non-animal cell, tissue or organ, in particular a plant cell, tissue or organ. The invention extends further to the use of said nucleotide sequences in producing non-animal cells, tissues and organs having a high bioavailable iron content than is present in otherwise isogenic lines which lack the subject nucleotide sequences or which contain said nucleotide sequences however do not express an iron-binding protein therefrom.

The invention extends further to the use of an isolated nucleic acid molecule as described herein to treat iron deficiency in a human or animal subject.

The hemoglobin or ferritin genetic sequence may be contained within a genetic construct, such as a plasmid, viral genome, viral sub-genomic fragment, bacteriophage, phagemid or cosmid molecule in a format suitable for expression in a non-animal cell, preferably a plant cell. Persons skilled in the art are aware of the requirements for producing such genetic constructs.

The present invention clearly extends to genetic constructs which comprise the complement of SEQ ID NOS:3 or 5 or homologues, analogues or derivatives thereof which may be readily derivable by those skilled in the art, such as a genomic gene equivalent, an RNA molecule, or synthetic DNA molecule, which at least comprises those nucleotide sequences which encode the iron-binding region of ferritin.

The genetic constructs used in the performance of the invention comprise, in addition to a genetic sequence which is capable of expressing an iron-binding protein or polypeptide, or a homologue, analogue or derivative thereof, a promoter and optional other regulatory sequences which modulate the expression of the subject genetic sequences. Such modulation of expression includes the conferring of an appropriate developmental, tissue-specific, cell-specific or organ-specific pattern of expression on the subject genetic sequence. It is also possible to modulate the expression of an iron-binding protein in response to external or environmental stimuli such as heat-shock, hypoxia, flooding, metal ions, antibiotic compounds, amongst others, such that expression of the iron-binding protein may be tightly regulated. Methods for modulation of gene expression are well-within the means of persons of ordinary skill in the art and require no undue experimentation on the part of such persons.

To produce a genetic construct which is useful for the present purpose means placing the genetic sequence which encodes an iron-binding protein, in particular the nucleotide sequence set forth in SEQ ID NO:1 or SEQ ID NO:3 or SEQ ID NO:5 or a homologue, analogue or derivative thereof, in operable connection with a promoter sequence which is capable of regulating the expression of the subject genetic sequence in a non-animal cell. Those skilled in the art will be aware that specific promoter sequences may be selected to

achieve the appropriate modulation of gene expression referred to *supra*.

In the present context, the term "promoter" is also used to describe a synthetic or fusion molecule, or derivative which confers, activates or enhances expression of the subject genetic sequence in a non-animal cell. Preferably, the promoter is capable of regulating expression of the genetic sequence in a plant cell, in particular a rice plant cell. Preferred promoters may contain additional copies of one or more specific regulatory elements, to further enhance expression or to alter the spatial expression and/or temporal expression of the genetic sequence which encodes an iron-binding protein. For example, regulatory elements which confer copper inducibility may be placed adjacent to a heterologous promoter sequence driving expression of the subject genetic sequence, thereby conferring copper inducibility on the expression of said molecule.

Placing a genetic sequence which encodes an iron-binding protein under the regulatory control of a promoter sequence means positioning the said genetic sequence such that expression is controlled by the promoter sequence. Promoters are generally positioned 5′ (upstream) to the genes that they control. In the construction of heterologous promoter/structural gene combinations it is generally preferred to position the promoter at a distance from the gene transcription start site that is approximately the same as the distance between that promoter and the gene it controls in its natural setting, i.e., the gene from which the promoter is derived. As is known in the art, some variation in this distance can be accommodated without loss of promoter function. Similarly, the preferred positioning of a regulatory sequence element with respect to a heterologous gene to be placed under its control is defined by the positioning of the element in its natural setting, i.e., the genes from which it is derived. Again, as is known in the art, some variation in this distance can also occur.

Examples of promoters suitable for use in genetic constructs of the present invention include viral-, fungal-, bacterial-, animal- and plant- derived promoters capable of functioning in non-animal cells, in particular plant cells such as those described *supra*.

In a preferred embodiment, however, the promoter is capable of expression in a monocotyledonous or dicotyledonous plant cell, for example a cell in a horticultural, vegetable, cereal or agricultural plant.

The promoter may regulate the expression of the said genetic sequence constitutively, or differentially with respect to the tissue in which expression occurs or, with respect to the developmental stage at which expression occurs, or in response to external stimuli such as physiological stresses, or plant pathogens, or metal ions, amongst others.

Examples of preferred promoters include the rice *Actin*1 promoter, rice ubiquitin gene promoter, rice glutelin promoter (i.e. GT1 promoter), wheat high molecular weight glutenin (HMW and/or Bx17) promoter, sucrose synthase promoter, CaMV 35S promoter, NOS promoter, octopine synthase (OCS) promoter, or *Arabidopsis thaliana SSU* gene promoter, amongst others.

In a particularly preferred embodiment, the promoter is the rice ubiquitin promoter, rice glutelin promoter, wheat HMW promoter or rice *Actin* 1 promoter sequence.

Optional additional regulatory sequences may be included in the genetic constructs, for example a terminator sequence placed 3′ or downstream of the subject genetic sequence.

The term "terminator" as used herein refers to a DNA sequence at the end of a transcriptional unit encoding an iron-binding protein, wherein said terminator signals termination of transcriptional. Terminators are 3′-non-translated DNA sequences containing a polyadenylation signal, which facilitates the addition of polyadenylate sequences to the 3′-end of a primary transcript. Terminators active in plant cells are known and described in the literature. They may be isolated from bacteria, fungi, viruses, animals and/or plants.

Examples of terminators particularly suitable for use in the genetic constructs of the present invention include the nopaline synthase (NOS) gene terminator of *Agrobacterium*

tumefaciens, the *Oryza sativa* ADP glucose pyrophosphorylase gene terminator (t3'bt2) the terminator of the Cauliflower mosaic virus (CaMV) 35S gene, the *zein* gene terminator from *Zea mays* or the Rubisco small subunit (SSU) gene terminator sequences, amongst others.

In a more particularly preferred embodiment, the terminator is the t3'bt2 terminator sequence.

Reference herein to a "promoter" is to be taken in its broadest context and includes the transcriptional regulatory sequences of a classical prokaryotic or eukaryotic genomic gene, including the TATA box which is required for accurate transcription initiation, with or without a CCAAT box sequence and additional regulatory elements (i.e. upstream activating sequences, enhancers and silencers) which alter gene expression in response to developmental and/or external stimuli, or in a tissue-specific manner. A promoter is usually, but not necessarily, positioned upstream or 5', of a structural gene, the expression of which it regulates. Furthermore, the regulatory elements comprising a promoter are usually positioned within 2 kb of the start site of transcription of the genetic sequence which encodes an iron-binding protein.

The level of expression of the genetic sequence encoding the iron-binding protein or polypeptide may be increased in a plant cell, tissue or organ, by including within the subject genetic construct a nucleotide sequence which comprises an intron derived from a eukaryotic gene and in particular, derived from a plant gene. Advantageously, the intron sequence is placed between the promoter sequence and the genetic sequence to which said promoter is operably connected.

In a particularly preferred embodiment, the intron sequence is derived from the shrunken-1 gene (.e. the *Sh1* intron). As exemplified herein, plant cells which express rice *A. thaliana* hemoglobin under the control of the GT-1 promoter sequence accumulate higher levels of hemoglobin if the sh1 intron sequence is placed downstream of the GT-1 promoter and upstream of the hemoglobin-encoding nucleotide sequence.

The present invention further contemplates the use of genetic constructs wherein the iron-binding protein is targeted to an organelle of a plant cell, such as a vacuole, plastid, mitochondria or the endoplasmic reticulum. In a particularly preferred embodiment, the *Arabidopsis thaliana* hemoglobin cDNA is placed operably under the control of the full-length high molecular weight glutenin promoter sequence wherein said promoter sequence contains a known transit peptide or organellar targeting sequence located at its 3'-end. Without being bound by any theory or mode of action, the recombinant hemoglobin peptide, polypeptide or protein is transported to an organelle in the transgenic plant where it is protected from the action of proteases and accumulates at a higher level than if expressed in the cytosol.

The genetic constructs of the invention may further include an origin of replication sequence which is required for replication in a specific cell type, for example a bacterial cell, when said genetic construct is required to be maintained as an episomal genetic element (e.g. plasmid or cosmid molecule) in said cell. Preferred origins of replication include, but are not limited to, the *f1*-ori and *col*E1 origins of replication.

The genetic construct may further comprise a selectable marker gene or genes that are functional in a cell into which said genetic construct is introduced.

As used herein, the term "selectable marker gene" includes any gene which confers a phenotype on a cell in which it is expressed to facilitate the identification and/or selection of cells which are transfected or transformed with a genetic construct of the invention or a derivative thereof.

Suitable selectable marker genes contemplated herein include the ampicillin resistance (Amp^r), tetracycline resistance gene (Tc), bacterial kanamycin resistance gene (Kan), phosphinothricin resistance gene, neomycin phosphotransferase gene (*npt*II), hygromycin resistance gene, β-glucuronidase (GUS) gene, chloramphenicol acetyltransferase (CAT) gene and luciferase gene, amongst others.

The genetic construct may be introduced into plant tissue, thereby producing a "transgenic plant", by various techniques known to those skilled in the art. The technique used for a given plant species or specific type of plant tissue depends on the known successful techniques. Means for introducing recombinant DNA into plant tissue include, but are not limited to, direct DNA uptake into protoplasts (Krens *et al*, 1982; Paszkowski *et al*, 1984), PEG-mediated uptake to protoplasts (Armstrong *et al*, 1990) microparticle bombardment electroporation (Fromm *et al.*, 1985), microinjection of DNA (Crossway *et al.*, 1986), microparticle bombardment of tissue explants or cells (Christou *et al*, 1988; Sanford, 1988) or T-DNA-mediated transfer from *Agrobacterium* to the plant tissue. Methods for the *Agrobacterium*-mediated transformation of plants will be well-known to those skilled in the art. In particular, methods for the *Agrobacterium*-mediated transformation of rice (*Oryza sativa*) tissue have been disclosed by Heie *et al.*, which is incorporated herein by way of reference. Representative T-DNA vector systems are described in the following references: An *et al.*(1985); Herrera-Estrella *et al.* (1983a,b); Herrera-Estrella *et al.* (1985).

For microparticle bombardment of cells, a microparticle is propelled into a plant cell, in particular a plant cell not amenable to *Agrobacterium* mediated transformation, to produce a transformed cell. Wherein the cell is a plant cell, a whole plant may be regenerated from the transformed plant cell. Alternatively, other non-animal cells derived from multicellular species may be regenerated into whole organisms by means known to those skilled in the art. Any suitable ballistic cell transformation methodology and apparatus can be used in practicing the present invention. Exemplary apparatus and procedures are disclosed by Stomp *et al.* (U.S. Patent No. 5,122,466) and Sanford and Wolf (U.S. Patent No. 4,945,050). When using ballistic transformation procedures, the genetic construct may incorporate a plasmid capable of replicating in the cell to be transformed.

Examples of microparticles suitable for use in such systems include 1 to 5 μm gold spheres. The DNA construct may be deposited on the microparticle by any suitable technique, such as by precipitation.

Plant species may be transformed with the genetic construct of the present invention by the DNA-mediated transformation of plant cell protoplasts and subsequent regeneration of the plant from the transformed protoplasts in accordance with procedures well known in the art.

Any plant tissue capable of subsequent clonal propagation, whether by organogenesis or embryogenesis, may be transformed with a vector of the present invention. The particular tissue chosen will vary depending on the clonal propagation systems available for, and best suited to, the particular species being transformed. Exemplary tissue targets include leaf disks, pollen, embryos, cotyledons, hypocotyls, megagametophytes, callus tissue, existing meristematic tissue (e.g., apical meristem, axillary buds, and root meristems), and induced meristem tissue (e.g., cotyledon meristem and hypocotyl meristem).

The term "organogenesis", as used herein, means a process by which shoots and roots are developed sequentially from meristematic centers.

The term "embryogenesis", as used herein, means a process by which shoots and roots develop together in a concerted fashion (not sequentially), whether from somatic cells or gametes.

Plants of the present invention may take a variety of forms. The plants may be chimeras of transformed cells and non-transformed cells; the plants may be clonal transformants (e.g., all cells transformed to contain the expression cassette); the plants may comprise grafts of transformed and untransformed tissues (e.g., a transformed root stock grafted to an untransformed scion in citrus species). The transformed plants may be propagated by a variety of means, such as by clonal propagation or classical breeding techniques. For example, a first generation (or T1) transformed plants may be selfed to give homozygous second generation (or T2) transformed plants, and the T2 plants further propagated through classical breeding techniques.

The genetic construct may further incorporate a dominant selectable marker, such as *npt*II, hygromycin-resistance gene, a phosphinothrium-resistance gene or ampicillin-resistance gene, amongst others, associated with the transforming DNA to assist in cell selection and breeding.

Plants which may be employed in practicing the present invention include all edible plants such as but not limited to, tobacco (*Nicotiana tabacum*), potato (*Solanum tuberosum*), soybean (*glycine max*), peanuts (*Arachis hypogaea*), cotton (*Gossypium hirsutum*), sweet potato (*Ipomoea batatus*), cassava (*Manihot esculenta*), coffee (*Cofea* spp.), coconut (*Cocos nucifera*), pineapple (*Ananas comosus*), citrus trees (*Citrus* spp.), cocoa (*Theobroma cacao*), tea (*Camellia sinensis*), banana (*Musa* spp.), avocado (*Persea americana*), fig (*Ficus casica*), guava (*Psidium guajava*), mango (*Mangifera indica*), olive (*Olea europaea*), papaya (*Carica papaya*), cashew (*Anacardium occidentale*), macadamia (*Macadamia integrifolia*), almond (*Prunus amygdalus*), sugar beets (*Beta vulgaris*), corn (*Zea mays*), wheat, oats, rye, barley, rice, vegetables, ornamentals, and conifers. Vegetables and pulses including tomatoes (*Lycopersicon esculentum*), lettuce (e.g., *Lactuea sativa*), green beans (*Phaseolus vulgaris*), lima beans (*Phaseolus limensis*), peas (*Pisum* spp.) and members of the genus *Cucumis* such as cucumber (*C. sativus*), cantaloupe (*C. cantalupensis*), and must melon (*C. melo*) may also be produced.

Once introduced into the plant tissue, the expression of the introduced gene may be assayed in a transient expression system, or it may be determined after selection for stable integration within the plant genome. Techniques are known for the *in vitro* culture of plant tissue, and in a number of cases, for regeneration into whole plants. Procedures for transferring the introduced genetic construct from the originally transformed plant into commercially useful cultivars are known to those skilled in the art.

The transgenic organisms, in particular transgenic plants and even more particularly transgenic rice plants which express an iron-binding protein, such as hemoglobin or ferritin, from a genetic construct as described herein are cultured, propagated or otherwise cultivated

on an appropriate medium comprising iron in the ferric or ferrous form, in addition to other macronutrients and micronutrients required for the maintenance of the said organism.

Accordingly, a further embodiment of the invention provides a method of increasing the bioavailable iron-content of a non-animal organism such as a plant, fungus, bacterium or algae, amongst others, said method comprising the steps of:

(i) introducing to said organism a genetic construct which comprises a genetic sequence which is capable of expressing an iron-binding protein; and

(ii) expressing said iron-binding protein in said organism,

wherein said second step is performed before, during or after culture, propagation or cultivation of said organism on a medium comprising a bioavailable concentration of iron.

The medium may be a liquid or solid medium, for example, wherein the transgenic organism is a plant, the medium may be a soil, compost mixture, hydroponic nutrient solution, Murashige and Skoog solid or liquid medium or other plant growth medium available to those skilled in the art.

The term "bioavailable concentration of iron" is used in the present context to indicate that the iron concentration present in the medium is such that, following uptake by the transgenic organism, the iron concentration delivered to the cell is sufficient to be bound by the iron-binding protein expressed therein.

Preferably, the concentration of iron of the medium is selected such that the concentration of iron delivered to the cell in which the subject iron-binding protein is expressed, is at least equal to the K_m of said iron-binding protein for iron, in the form in which it is contained in the medium, or equal to the K_m of the iron-binding protein for heme-iron. More preferably, the iron concentration delivered to the cell is 2-fold the K_m of the iron-binding protein for iron in the form in which it is contained in the medium or heme-iron. Even more preferably, the iron concentration delivered to the cell is sufficient to fully saturate the iron-binding protein with iron in the form in which it is present in the medium, or to fully

saturate the iron-binding protein with heme-iron.

A further aspect of the present invention provides a transgenic non-animal organism having a high bioavailable iron-content wherein said organism has been produced according to the methods described herein.

Accordingly, the transgenic non-animal organism of the invention contains a significantly higher level of iron, present as heme-iron or free Fe^{2+} or Fe^{3+}, than do isogenic isolates of the same organism which do not express the foreign iron-binding protein introduced thereto.

Preferably, the transgenic non-animal organism is a plant, more preferably a rice plant. Other plants are not excluded .

The present invention extends to the progeny cells, tissues, organs and organisms of the said transgenic non-animal organism.

The transgenic non-animal organism may be administered to a human or animal subject as food or in feed or it may be processed to produce a medicated foodstuff suitable for administration to a human or other animal suffering from an iron deficiency, as a result of inadequate dietary iron intake, medical illness or menstruation, amongst other conditions.

Accordingly, a further aspect of the invention contemplates a medicated foodstuff suitable for the treatment of iron-deficiency in humans or other animals wherein said medicated foodstuff comprises iron as an active compound derived from a transgenic non-animal organism produced according to any of the embodiments described herein.

A further aspect of the present invention contemplates a method of treating an iron deficiency in a human or non-human animal, said method comprising administering to a human or non-human animal suffering from said iron-deficiency a medicated foodstuff

produced essentially as described herein.

Medicated foodstuffs prepared from the genetically-transformed non-animal organism may be administered orally or injected in any convenient manner.

Oral administration is usually in the form of a liquid iron tonic, a capsule, tablet or powder.

The active compounds may also be administered in dispersions prepared in glycerol, liquid polyethylene glycols, and/or mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms.

The pharmaceutical forms suitable for parenteral administration include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. In all cases the form must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thirmerosal and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example.

Sterile injectable solutions are prepared by incorporating the iron derived from the non-animal transgenic organism in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filter sterilisation. Generally, dispersions are prepared by incorporating the various sterilised active ingredient(s) into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and the freeze-drying technique which yield a powder of the active ingredient plus any additional desired ingredient from previously sterile-filtered solution thereof.

Carriers and/or diluents suitable for veterinary use include any and all solvents, dispersion media, aqueous solutions, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, use thereof in the composition is contemplated. Supplementary active ingredients can also be incorporated into the compositions.

A still further aspect of the present invention provides an isolated ferritin gene which comprises a sequence of nucleotides or is complementary to a sequence of nucleotides substantially as set forth in SEQ ID NO:3 or a homologue, analogue or derivative thereof which is at least about 85% identical to SEQ ID NO:3 or a complementary sequence thereto.

Preferably, the percentage similarity to a sequence set forth in SEQ ID NOS:1 or 3 is at least 90%. Even more preferably, the percentage identity is at least 95%. Still more preferably, the percentage identity is at least 99%.

Reference herein to a "gene" is to be taken in its broadest context and includes:

(i) a classical genomic gene consisting of a coding region optionally together with transcriptional and/or translational regulatory sequences and a coding region with or without

non-translated sequences (i.e. introns, 5′- and 3′- untranslated sequences); and/or

(ii) mRNA or cDNA corresponding to the coding regions (i.e. exons) and optionally 5′- and 3′- untranslated sequences of the gene; and/or

(iii) EST sequences capable of hybridising to said genomic gene, cDNA or mRNA which are at least capable of encoding an iron-binding polypeptide.

The term "gene" is also used to describe a synthetic or fusion molecule, or derivative which encodes, or is complementary to a molecule which encodes, all or part of a functional product. In the present context, a functional product is a protein or polypeptide or derivative thereof which is capable of binding iron and, as a consequence, is useful for the purpose of the present invention as stated herein.

The genetic sequences which encode rice ferritin may correspond to the naturally occurring sequence or may differ by one or more nucleotide substitutions, deletions and/or additions. Accordingly, the present invention extends to iron-binding genes and any functional genes, mutants, derivatives, parts, fragments, homologues or analogues thereof.

Preferred genes according to this aspect of the invention are capable of encoding an iron-binding polypeptide. Preferred homologues, analogues and derivatives of the rice ferritin cDNA sequence set forth in SEQ ID NO:3 will be derived from a naturally occurring ferritin genes by standard recombinant techniques. Generally, the rice ferritin gene may be subjected to mutagenesis to produce single or multiple nucleotide substitutions, deletions and/or additions.

Nucleotide insertional derivatives of the iron-binding gene of the present invention include 5′ and 3′ terminal fusions as well as intra-sequence insertions of single or multiple nucleotides. Insertional nucleotide sequence variants are those in which one or more nucleotides are introduced into a predetermined site in the nucleotide sequence although random insertion is also possible with suitable screening of the resulting product. Deletional variants are characterised by the removal of one or more nucleotides from the sequence. Substitutional nucleotide variants are those in which at least one nucleotide in the sequence

has been removed and a different nucleotide inserted in its place. Such a substitution may be "silent" in that the substitution does not change the amino acid defined by the codon. Alternatively, substituents are designed to alter one amino acid for another similar acting amino acid, or amino acid of like charge, polarity, or hydrophobicity.

The present invention extends to the isolated nucleic acid encoding rice ferritin when integrated into a plant genome and to propagated plants derived therefrom which comprise the introduced rice ferritin gene.

In an alternative embodiment, the present invention contemplates a nucleic acid molecule which encodes an iron-binding protein and which is capable of hybridising under at least moderate or high stringency conditions to the nucleic acid molecule set forth in SEQ ID NO: 3 or a complementary sequence thereto.

The present invention is further described by the following non-limiting Examples.

EXAMPLE 1

MANIPULATION OF HEMOGLOBIN EXPRESSION IN RICE SEEDS BY GENETIC ENGINEERING

I. Construction of plasmids containing the *Arabidopsis* hemoglobin cDNA driven by a range of promoters

Rice (Hb Rice) and the *Arabidopsis thaliana* hemoglobin (HbAra) cDNAs were inserted into constructs *suit*able for introduction into rice by particle gun bombardment or *Agrobacterium*-mediated transformation. The two sequences were placed downstream of constitutive promoters (the promoter of the rice Actin gene and the promoter of the rice Ubiquitin gene, or a seed-specific truncated rice glutelin promoter (GT1) with a Sh1 intron,

the full length GT1 promoter, a truncated wheat high molecular weight glutenin promoter (HMW) and the full length wheat high molecular weight glutenin promoter (Bx17).

Plasmids which express the rice hemoglobin (HbRice) protein under the control of the glutelin, *Actin*1 or HMW promoter sequences are shown in Figures 2, 4 and 6, respectively. In Figure 7, the genetic construct comprises, in addition to the GT1 promoter sequence, the intron 1 sequence of the maize shrunken1 gene.

All plasmids comprising the HbAra sequence utilised the nopaline synthase terminator gene terminator of *Agrobacterium tumefaciens* (tNOS) or the *Oryza sativa* ADP glucose pyrophosphorylase gene terminator (t3'Bt2).

The plasmids set forth in Figures 1 to 3 and Figures 5 to 8 were constructed using standard procedures according to the schematic representations presented in Figures 9 to 15 inclusive.

II. Production of transgenic plants

The hemoglobin constructs were introduced into rice *via* particle bombardment and, in the case of the construct containing *A. thaliana* hemoglobin under control of the Bx17 promoter, using *Agrobacterium*-mediated transformation procedures. Plants were regenerated. The number of lines obtained for each construct is given in Table 3.

TABLE 3

Numbers of transgenic rice lines containing genetic constructs encoding a plant hemoglobin produced in 1996 and 1997

Transformation Method and Construct Name	N° lines obtained per experiment in each year	
	1996	1997
Particle gun:		
ActI-HbAra	6	
ActI-HbRice	5	
HMW-HbAra	3	
HMW-HbRice	17	
GT1-HbAra	3	7
Ubiq-HbAra		33
Bx17-HbAra		22
GT1-sh1-HbAra		29
***Agrobacterium*:**		
Bx17-HbAra		25

Callus were also generated from mature seed of the Australian rice cultivars Jarrah, Millin and YRM13 for transformation with the *Arabidopsis thaliana* hemoglobin cDNA, placed operably under the control of the full-length wheat high molecular weight glutenin promoter sequence. Taipei was used as a control for the transformation and regeneration efficiencies. A genetic construct comprising the *A. thaliana* hemoglobin cDNA operably under control of the full-length HMW promoter was introduced into callus by particle bombardment or *Agrobacterium*-mediated transformation. In a preliminary experiment, plants were regenerated from Jarrah calli which had been bombarded with this genetic construct.

III. Analysis of the transgenic plants

The expression of the endogenous hemoglobin was investigated in untransformed rice plants by western blotting. No hemoglobin protein was detected in leaves. Low levels of hemoglobin protein were found in seeds.

Gene copy number and number of loci containing the introduced gene was investigated in T_o plant lines by Southern hybridization and results are shown in Figure 21 for rice hemoglobin constructs. Southern hybridization was also used to detect gene copy number in segregating T_1 plant lines (Figure 22). The protein content of seeds coming from the 1996 transgenic plants (T1 seeds) was analysed by western blotting (Figure 23) and an increase in hemoglobin levels was detected immunologically using antibodies against the *Arabidopsis* hemoglobin. A summary of characterizing data for the hemoglobin - overexpressing lines is presented in Table 4.

T1 seeds from overexpressing lines were germinated, and after 5 months seeds from those plants were collected (T2 seeds). For each plant, proteins were extracted from 20 seeds and their individual protein content analysed immunologically using antibodies against hemoglobin. Using this method homozygous transgenic lines were identified.

The level of hemoglobin in the seeds of transgenic homozygous and heterozygous lines was estimated using western blotting (Figure 24). By comparing the expression level of hemoglobin in homozygous lines against a hemoglobin protein standard (Figure 25), it was possible to quantitate the expression level (Figure 26). Highest seed expression levels of hemoglobin were observed for homozygous rice lines expressing *A. thaliana* hemoglobin under control of the GT1 promoter sequence. Transgenic lines were found to contain 0.5 to 1.5 ng of hemoglobin per μg of soluble seed proteins. These quantities are 15 to 85 times greater than what is present in non transformed seeds (Figure 5). The most effective promoter was the full length rice glutelin promoter (1.5 ng of hemoglobin per μg of soluble seed proteins).

TABLE 4

Characterisation of hemoglobin-overexpressing lines

Lines	Expression levels (T1 seeds)	Gene copy number (T0 plants)	Loci number (T0 plants)
HMW-HbRice			
7.20.1	+	5	1
7.12.1	+	10	8
7.10.1	+	6	1
7.8.1	+	4	3
7.14.1	+++	2	1
7.5.1	++++	5	2
7.9.1	++++	2	1
HMW-HbAra			
6.1.1	++++	4	2
6.3.1	++++	4	2
GT1-HbAra			
9.1	++++++	1	1
Act-HbAra			
5.3.1	+++	2	1
Act-HbRice			
4.1.2	+	2	
4.21.1	+	2	

EXAMPLE 2

MANIPULATION OF FERRITIN EXPRESSION IN RICE SEEDS BY GENETIC ENGINEERING

I. Cloning of the rice ferritin cDNA

The rice ferritin cDNA was identified by searching the Rice Expressed Sequence Tag (EST) database. The single partial length cDNA was showing a strong homology to maize ferritin 1 cDNA (FM1; SEQ ID NO:5) and maize ferritin 2 cDNA (FM2). An IR54 cDNA library from anaerobically-treated cells was screened using the EST as a probe and allowed the isolation of a full length rice ferritin cDNA (OSFer; SEQ ID NO:3). The FM1 sequence is 1139 nucleotides long (ATG-polyA sequence) and contain an open reading frame of 254 amino acids. A 3' untranslated region of 380 nucleotides is found downstream of a stop codon and a putative polyA+tail was found. The sequence of a putative chloroplast-type transit peptide has been identified (amino acid residues 1 to 43). The OSfer sequence is 987 nucleotides long and contain an open reading frame of 197 amino acids. Osfer ferritin protein has 83.85% identity with the maize ferritin protein (Figure 27).

II. Construction of plasmids containing the rice ferritin cDNA driven by a range of promoters

The OSfer cDNA was modified by PCR: a second ATG was introduced in frame with the endogenous one. BamH1 sites were introduced just before the ATG and just before the beginning of the putative poly A+tail (OSfer long) or just after the stop codon (OSfer short). OSfer long and OSfer short were inserted into constructs suitable for introduction into rice by particle gun bombardment or *Agrobacterium*-mediated transformation. The two sequences were placed downstream of a constitutive promoter (the promoter of the rice Ubiquitin gene) and seed-specific promoters: a truncated rice glutelin promoter (GT1) with a *Sh1* intron, the full length GT1 promoter, a truncated wheat high molecular weight glutenin promoter (HMW) and the full length wheat high molecular weight glutening promoter (Bx17).

III. Transformation of rice with ferritin constructs

The ferritin constructs were introduced into rice *via* particle bombardment. Plants were regenerated. The number of lines obtained for each construct is given in Table 5.

TABLE 5

Results of the transformation of rice with the ferritin constructs

Construct	N° lines
Bx17-OSfer short	4
Bx17-OSfer long	7
HMW-OSfer short	8
HMW-OSfer long	7
Ubiq-OSfer short	-
Ubiq-OSfer long	11
pGT1-OSfer short	7
pGT1-OSfer long	11
pGT1-sh1-OSfer short	5
pGT1-sh1-OSfer long	6

IV. Expression of the ferritin in wild type and transgenic plants

Antibodies were tested on 9 young T_0 plants which were transformed using the Ubiquitin-OSfer short construct and 2 untransformed young plants. Strong ferritin expression was detected in many of the protein extracts from untransformed or transformed plants (Figure 28). Two transformed lines showed less ferritin expression than the control lines. The same experiment was done again using 3 controls and 11 T0 plants which were transformed using the Ubiquitin-OSfer short construct (Figure 29). A high variability was observed among the controls and the transformed plants. To determine where and when endogenous ferritin levels are high, proteins were extracted from the tip of leaves, roots and seeds of untransformed plants. Ferritin seems to be expressed at high levels in the tip of rice

leaves, independently of the age of the leaves. It is also expressed, at lower levels, in dry rice seeds and roots (Figures 30 and 31).

EXAMPLE 3

CORRECTION OF IRON DEFICIENCIES IN A RAT MODEL

Rats are maintained on an iron-deficient diet for 2-3 weeks. Approximately 2.5 kilograms of transgenic rice seed over-expressing recombinant rice ferritin or hemoglobin is collected and fed to 6 iron-deficient rats, each consuming 20g feed per day comprising 70% rice balance, over a total period of 4 weeks. Control lines of iron-deficient rats are fed on untransformed, otherwise isogenic rice seed. Hemoglobin repletion is assayed after 4 weeks to determine the effect of the transgenic rice diet on overcoming iron-deficiency.

EXAMPLE 4

CORRECTION OF IRON DEFICIENCIES IN A PIG MODEL

Pigs are naturally anaemic animals and normally receive an iron injection at birth. The gastrointestinal tract of the pig is similar to the human gastrointestinal tract and pigs will consume a diet typical of that eaten by humans. As a consequence, the pig is a suitable model for assessing the bioavailability of iron in a human diet.

A population of pigs is maintained on an iron-deficient diet until anaemic. Transgenic rice seed over-expressing recombinant rice ferritin or hemoglobin is collected and fed to iron-deficient pigs, each consuming a diet comprising 70% rice balance, over a total period of about 4 weeks. Control anaemic animals are fed on untransformed, otherwise isogenic rice seed. A second population of control non-anaemic animals is also fed on the transformed rice seed or untransformed rice seed. Hemoglobin repletion is assayed after 4 weeks to determine the effect of the transgenic rice diet on overcoming anaemia. Additionally, tissue samples and gut contents are analysed.

EXAMPLE 5

RECOMBINANT PRODUCTION OF RICE FERRITIN IN *E. coli AND* GENERATION OF ANTI-RICE FERRITIN ANTIBODIES

OSfer short was inserted into an expression vector pQE30. The 6xHis coding sequence was placed in frame at the 5' end of OSfer short. After expression in *E.coli*, the recombinant protein was expressed and purified (Figure 32). It was then used to generate rabbit anti-OSfer.

EQUIVALENTS

Those skilled in the art will be aware that the present invention is subject to variations and modifications other than those specifically described herein. It is to be understood that the invention includes all such variations and modifications. The invention also includes all such steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:COMMONWEALTH SCIENTIFIC AND INDUSTRIAL RESEARCH ORGANISATION

(ii) TITLE OF INVENTION: METHOD OF INCREASING THE IRON CONTENT OF PLANT CELLS

(iii) NUMBER OF SEQUENCES: 6

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: DAVIES COLLISON CAVE
(B) STREET: 1, LITTLE COLLINS STREET
(C) CITY: MELBOURNE
(D) STATE: VICTORIA
(E) COUNTRY: AUSTRALIA
(F) ZIP: 3000

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

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(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: JOHN M. SLATTERY
(B) REGISTRATION NUMBER:
(C) REFERENCE/DOCKET NUMBER:

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 61 3 9254 2777
(B) TELEFAX: 61 3 9254 2770

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 900 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Arabidopsis thaliana

(ix) FEATURE:
(A) NAME/KEY: exon
(B) LOCATION: 1..171

(ix) FEATURE:
(A) NAME/KEY: intron
(B) LOCATION: 172..253

(ix) FEATURE:
(A) NAME/KEY: exon
(B) LOCATION: 254..367

(ix) FEATURE:
(A) NAME/KEY: intron
(B) LOCATION: 368..446

(ix) FEATURE:
(A) NAME/KEY: exon
(B) LOCATION: 447..566

(ix) FEATURE:
(A) NAME/KEY: intron
(B) LOCATION: 567..663

(ix) FEATURE:
(A) NAME/KEY: exon
(B) LOCATION: 664..900

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 59..172

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 255..368

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(A) NAME/KEY: CDS
(B) LOCATION: 448..567

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 665..796

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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ggagatgaag gtggcttggg gtcaggctta tgatcacctt gttgctgcca ttaaagctga 780

aatgaatctt tccaactaaa aaatcatata ctattatata gttgtaaact tgtaataaat 840

atttcatttt gaattgttct catgactgtt gttctatttg gtttggtttg atttagtgac 900

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 160 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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1 5 10 15

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20 25 30

Leu Lys Leu Phe Ile Lys Ile Phe Glu Ile Ala Pro Thr Thr Lys Lys
35 40 45

Met Phe Ser Phe Leu Arg Asp Ser Pro Ile Pro Ala Glu Gln Asn Pro
50 55 60

Lys Leu Lys Pro His Ala Met Ser Val Phe Val Met Cys Cys Glu Ser
65 70 75 80

Ala Val Gln Leu Arg Lys Thr Gly Lys Val Thr Val Arg Glu Thr Thr
85 90 95

Leu Lys Arg Leu Gly Ala Ser His Ser Lys Tyr Gly Val Val Asp Glu
100 105 110

His Phe Glu Val Ala Lys Tyr Ala Leu Leu Glu Thr Ile Lys Glu Ala
115 120 125

Val Pro Glu Met Trp Ser Pro Glu Met Lys Val Ala Trp Gly Gln Ala
130 135 140

Tyr Asp His Leu Val Ala Ala Ile Lys Ala Glu Met Asn Leu Ser Asn
145 150 155 160

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 987 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Oryza sativa

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..591

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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 1               5                  10                  15

tat ctc gcc gcc gcg gcc tcg acc cct gct tcc gtc tgg ctg cct gtg      96
Tyr Leu Ala Ala Ala Ala Ser Thr Pro Ala Ser Val Trp Leu Pro Val
            20                  25                  30

ccg cgt ggt gcc gga ccc ggg gca gtg tgc agg gcc gcc ggg aaa ggg     144
Pro Arg Gly Ala Gly Pro Gly Ala Val Cys Arg Ala Ala Gly Lys Gly
        35                  40                  45

aag gag gtg ctc agc ggc gtg gtc ttc cag cca ttc gag gag ctc aag     192
Lys Glu Val Leu Ser Gly Val Val Phe Gln Pro Phe Glu Glu Leu Lys
    50                  55                  60

ggg gag ctc tcc ctc gtc ccc cag gcc aag gac cag tct ctc gct agg     240
Gly Glu Leu Ser Leu Val Pro Gln Ala Lys Asp Gln Ser Leu Ala Arg
65                  70                  75                  80
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gca aaa ttc gtc gac gag tgc gag gcc gcc atc aac gag cag atc aat      288
Ala Lys Phe Val Asp Glu Cys Glu Ala Ala Ile Asn Glu Gln Ile Asn
                85                  90                  95

gtg gag tac aat gca tct tac gcg tac cac tcc ctt ttc gcc tac ttt      336
Val Glu Tyr Asn Ala Ser Tyr Ala Tyr His Ser Leu Phe Ala Tyr Phe
            100                 105                 110

gac cgt gac aac gtt gct ctc aag gga ttc gcc aaa ttc ttc aaa gaa      384
Asp Arg Asp Asn Val Ala Leu Lys Gly Phe Ala Lys Phe Phe Lys Glu
        115                 120                 125

tcc agc gat aat gag aag ttg cac aac ctg cac agt gtg gca tca agg      432
Ser Ser Asp Asn Glu Lys Leu His Asn Leu His Ser Val Ala Ser Arg
    130                 135                 140

tgc aat gat cca cag ctg acc gat tcg ttt gag agc gaa ttc ctt gag      480
Cys Asn Asp Pro Gln Leu Thr Asp Ser Phe Glu Ser Glu Phe Leu Glu
145                 150                 155                 160

gag cag gtt gaa gcc atc aag aag atc tct gag tat gtc gcc cag ctg      528
Glu Gln Val Glu Ala Ile Lys Lys Ile Ser Glu Tyr Val Ala Gln Leu
                165                 170                 175

aga aga gtg gga aag ggg cat ggg gtg tgg cac ttt gan nag aag ctg      576
Arg Arg Val Gly Lys Gly His Gly Val Trp His Phe Xaa Xaa Lys Leu
            180                 185                 190

ctt gag gaa gaa gct tgaatggagg agacggtgtg aagggcagta gtaggtttcg      631
Leu Glu Glu Glu Ala
        195

tttcttccat ccatgggatc atgcagctaa agaaagaaaa taagtcgagt ctgtgtattc    691

atcaaattaa gcacgcagta gcaatggagt gaatgaacca accaatttgg tcatgaactt    751

ccccctgtgt ctaggtcagt agaagcttca gaatcatgtg tatgtcgcgc gtcctcgcag    811

aaaaggaacn atgngcgcgg ctggctgtag cgatacttgt gatattgtgg ctatgcatgg    871
```

agtcatggtg atggggaatt ttgtggagca tttacacgtt tgttgctaga gagattttgc 931

tgggcagcca ataaggaaca gtatgataaa aaaaaaaaaa aaaggcggcc gccaac 987

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 197 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Met Leu Pro Pro Arg Val Ala Pro Ala Ala Ala Ala Ala Ala Pro Thr
1 5 10 15

Tyr Leu Ala Ala Ala Ala Ser Thr Pro Ala Ser Val Trp Leu Pro Val
20 25 30

Pro Arg Gly Ala Gly Pro Gly Ala Val Cys Arg Ala Ala Gly Lys Gly
35 40 45

Lys Glu Val Leu Ser Gly Val Val Phe Gln Pro Phe Glu Glu Leu Lys
50 55 60

Gly Glu Leu Ser Leu Val Pro Gln Ala Lys Asp Gln Ser Leu Ala Arg
65 70 75 80

Ala Lys Phe Val Asp Glu Cys Glu Ala Ala Ile Asn Glu Gln Ile Asn
85 90 95

Val Glu Tyr Asn Ala Ser Tyr Ala Tyr His Ser Leu Phe Ala Tyr Phe
100 105 110

Asp Arg Asp Asn Val Ala Leu Lys Gly Phe Ala Lys Phe Phe Lys Glu
115 120 125

Ser Ser Asp Asn Glu Lys Leu His Asn Leu His Ser Val Ala Ser Arg
130 135 140

Cys Asn Asp Pro Gln Leu Thr Asp Ser Phe Glu Ser Glu Phe Leu Glu
145 150 155 160

Glu Gln Val Glu Ala Ile Lys Lys Ile Ser Glu Tyr Val Ala Gln Leu
165 170 175

Arg Arg Val Gly Lys Gly His Gly Val Trp His Phe Xaa Xaa Lys Leu
180 185 190

Leu Glu Glu Glu Ala
195

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1292 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Zea mays

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 94..858

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

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gcgaacgcac acgcacccac acgacacatc cag atg atg ctt agg gtt tcc ccg      114
                                    Met Met Leu Arg Val Ser Pro
                                      1               5

tcc ccg gcc gcc gcg gtg cca acc cag ctc tcc ggt gcg ccc gcg acc      162
Ser Pro Ala Ala Ala Val Pro Thr Gln Leu Ser Gly Ala Pro Ala Thr
         10                  15                  20

cct gct ccc gtc gtc agg gtg gcg gcg ccg cgc gga gtc gcc tcg ccg      210
Pro Ala Pro Val Val Arg Val Ala Ala Pro Arg Gly Val Ala Ser Pro
     25                  30                  35

tcc gct ggc gcc gcg tgc agg gcc gcg ggc aag ggg aag gag gtg ctc      258
Ser Ala Gly Ala Ala Cys Arg Ala Ala Gly Lys Gly Lys Glu Val Leu
 40                  45                  50                  55

agc ggg gtg gtg ttc cag ccc ttc gag gag atc aag ggg gag ctc gcc      306
Ser Gly Val Val Phe Gln Pro Phe Glu Glu Ile Lys Gly Glu Leu Ala
                 60                  65                  70

ctc gtg ccc cag tcc cca gac aag tcg ctc gcg cgc cac aag ttc gtc      354
Leu Val Pro Gln Ser Pro Asp Lys Ser Leu Ala Arg His Lys Phe Val
             75                  80                  85

gat gac tgc gag gcc gca ctc aac gag cag atc aac gtg gag tac aac      402
Asp Asp Cys Glu Ala Ala Leu Asn Glu Gln Ile Asn Val Glu Tyr Asn
         90                  95                 100

gcc tcg tat gca tac cac tcc ctc ttc gcc tat ttc gac cgc gac aac      450
Ala Ser Tyr Ala Tyr His Ser Leu Phe Ala Tyr Phe Asp Arg Asp Asn
     105                 110                 115

gtg gct ctc aaa gga ttt gcc aag ttc ttc aag gaa tcc agc gac gag      498
Val Ala Leu Lys Gly Phe Ala Lys Phe Phe Lys Glu Ser Ser Asp Glu
 120                 125                 130                 135
```

```
gag agg gag cac gct gaa aag ctc atg gag tac cag aac aaa cgt gga      546
Glu Arg Glu His Ala Glu Lys Leu Met Glu Tyr Gln Asn Lys Arg Gly
                140                 145                 150

ggc agg gtg agg ctc caa tcg att gtg acg cct tta acc gag ttt gac      594
Gly Arg Val Arg Leu Gln Ser Ile Val Thr Pro Leu Thr Glu Phe Asp
            155                 160                 165

cac cct gag aaa gga gat gct ctg tac gct atg gag ctg gct ctg gcc      642
His Pro Glu Lys Gly Asp Ala Leu Tyr Ala Met Glu Leu Ala Leu Ala
        170                 175                 180

ctg gaa aag ctg gtc aat gaa aag ctg cac aac ctg cat ggt gtg gca      690
Leu Glu Lys Leu Val Asn Glu Lys Leu His Asn Leu His Gly Val Ala
    185                 190                 195

aca agg tgc aat gat cct cag ctg aca gac ttc atc gag agt gag ttc      738
Thr Arg Cys Asn Asp Pro Gln Leu Thr Asp Phe Ile Glu Ser Glu Phe
200                 205                 210                 215

ctc gag gag cag ggt gaa gcc ata aac aag atc tcc aag tat gtc gcc      786
Leu Glu Glu Gln Gly Glu Ala Ile Asn Lys Ile Ser Lys Tyr Val Ala
                220                 225                 230

cag ctg agg aga gtg ggc aag ggg cac ggg gtg tgg cac ttc gat cag      834
Gln Leu Arg Arg Val Gly Lys Gly His Gly Val Trp His Phe Asp Gln
            235                 240                 245

atg ctg ctt gag gaa gag gcc taaaaagaag gaacggacag atgaatgaag        885
Met Leu Leu Glu Glu Glu Ala
        250                 255

gaaacaagtg tgtaatgttg atcaggcgga ggaataatgg gtcaactgac aactctgggt    945

cggtggattt gtagttggtt tgggtttttt agttgtagaa cggtgttgcc cccctcctag   1005

agagggatga acacattatg catgcgatgt gtcagttcgg tcggtgctat gctcgttggg   1065

atttgattgt cggaaataaa acatgggtga tgaatgtgta ccctccctaa tctatgtctt   1125
```

ggtgatgaag ttgagaaagc tgcgggtgtt gtaggtggat ttgtagagtt ttttcttcag 1185

ttggttaggt tcgctgttgc ctacctcatt tgcccctcat catcgccgta atttatttat 1245

ttataatata aaagatccag attttccccc aaaaaaaaaa aaaaaaa 1292

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 254 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Met Met Leu Arg Val Ser Pro Ser Pro Ala Ala Ala Val Pro Thr Gln
1 5 10 15

Leu Ser Gly Ala Pro Ala Thr Pro Ala Pro Val Val Arg Val Ala Ala
20 25 30

Pro Arg Gly Val Ala Ser Pro Ser Ala Gly Ala Ala Cys Arg Ala Ala
35 40 45

Gly Lys Gly Lys Glu Val Leu Ser Gly Val Val Phe Gln Pro Phe Glu
50 55 60

Glu Ile Lys Gly Glu Leu Ala Leu Val Pro Gln Ser Pro Asp Lys Ser
65 70 75 80

Leu Ala Arg His Lys Phe Val Asp Asp Cys Glu Ala Ala Leu Asn Glu
85 90 95

Gln Ile Asn Val Glu Tyr Asn Ala Ser Tyr Ala Tyr His Ser Leu Phe
100 105 110

Ala Tyr Phe Asp Arg Asp Asn Val Ala Leu Lys Gly Phe Ala Lys Phe

115 120 125

Phe Lys Glu Ser Ser Asp Glu Glu Arg Glu His Ala Glu Lys Leu Met
130 135 140

Glu Tyr Gln Asn Lys Arg Gly Gly Arg Val Arg Leu Gln Ser Ile Val
145 150 155 160

Thr Pro Leu Thr Glu Phe Asp His Pro Glu Lys Gly Asp Ala Leu Tyr
165 170 175

Ala Met Glu Leu Ala Leu Ala Leu Glu Lys Leu Val Asn Glu Lys Leu
180 185 190

His Asn Leu His Gly Val Ala Thr Arg Cys Asn Asp Pro Gln Leu Thr
195 200 205

Asp Phe Ile Glu Ser Glu Phe Leu Glu Glu Gln Gly Glu Ala Ile Asn
210 215 220

Lys Ile Ser Lys Tyr Val Ala Gln Leu Arg Arg Val Gly Lys Gly His
225 230 235 240

Gly Val Trp His Phe Asp Gln Met Leu Leu Glu Glu Glu Ala
245 250

CLAIMS:

1. A method of increasing the bioavailable iron content of a non-animal organism, organ, tissue or cell, said method comprising the steps of introducing thereto a genetic sequence which encodes an iron-binding protein and expressing said protein therein for a time and under conditions sufficient for the level of said iron-binding protein to increase.

2. The method according to claim 1, wherein the non-animal organism, organ, tissue or cell is a plant, plant organ, plant tissue or plant cell.

3. The method according to claim 2, wherein the plant, plant organ, plant tissue or plant cell in which expression occurs comprises or is derived from an edible plant or plant part.

4. The method according to claim 3, wherein the edible plant or plant part is a cereal plant or a cereal plant part.

5. The method according to claims 3 or 4, wherein the plant part is seed.

6. The method according to claim 4, wherein the cereal plant is rice or the cereal plant part is derived from rice.

7. The method according to any one of claims 1 to 6, wherein the iron-binding protein is a non-animal hemoglobin peptide, polypeptide or protein.

8. The method according to claim 7, wherein the non-animal hemoglobin peptide, polypeptide or protein comprises a sequence of amino acids which is at least about 85% similar to the *Arabidopsis thaliana* hemoglobin amino acid sequence set forth in SEQ ID NO:2 or a homologue, analogue or derivative thereof having the capacity to bind iron.

9. The method according to any one of claims 1 to 6, wherein the iron-binding protein is

a non-animal ferritin peptide, polypeptide or protein.

10. The method according to claim 9, wherein the non-animal ferritin peptide, polypeptide or protein is derived from a plant.

11. The method according to claim 10, wherein the non-animal ferritin peptide, polypeptide or protein is derived from a cereal plant.

12. The method according to claim 11, wherein the non-animal ferritin peptide, polypeptide or protein is derived from a maize plant or a rice plant.

13. The method according to claim 12, wherein the ferritin peptide, polypeptide or protein comprises a sequence of amino acids which is at least about 85% similar to the rice ferritin amino acid sequence set forth in SEQ ID NO:4 or a homologue, analogue or derivative thereof having the capacity to bind iron.

14. The method according to claim 12, wherein the ferritin peptide, polypeptide or protein comprises a sequence of amino acids which is at least about 85% similar to the maize ferritin amino acid sequence set forth in SEQ ID NO:6 or a homologue, analogue or derivative thereof having the capacity to bind iron.

15. The method according to claim 1, wherein the genetic sequence which encodes the iron-binding protein comprises a nucleotide sequence which is at least about 85% identical to the nucleotide sequence of the *Arabidopsis thaliana* hemoglobin gene set forth in SEQ ID NO:1 or a homologue, analogue or derivative thereof which is capable of encoding an iron-binding peptide, polypeptide or protein..

16. The method according to claim 1, wherein the genetic sequence which encodes the iron-binding protein comprises a nucleotide sequence which is at least about 85% identical to the nucleotide sequence of the rice ferritin cDNA sequence set forth in SEQ ID NO:3 or a

homologue, analogue or derivative thereof which is capable of encoding an iron-binding peptide, polypeptide or protein.

17. The method according to claim 1, wherein the genetic sequence which encodes the iron-binding protein comprises a nucleotide sequence which is at least about 85% identical to the maize ferritin cDNA sequence set forth in SEQ ID NO:5 or a homologue, analogue or derivative thereof which is capable of encoding an iron-binding peptide, polypeptide or protein..

18. The method according to claim 1, wherein the genetic sequence which encodes the iron-binding protein is contained within a genetic construct which is suitable for introduction into a cell, tissue or organ in which expression is required and maintenance in said cell, tissue or organ and wherein said genetic sequence is further operably connected in said genetic construct to the following sequences:

(i) a promoter sequence capable of regulating expression of said genetic sequence in a cell, tissue or organ in which expression of the iron-binding protein is required; and

(ii) a transcription termination sequence placed at the 3′-end or downstream of the coding region of said genetic sequence.

19. The method according to claim 18, wherein the iron-binding protein is a plant-derived ferritin or hemoglobin peptide, polypeptide or protein.

20. The method according to claims 18 or 19, wherein the promoter sequence is a plant-expressible promoter sequence.

21. The method according to claim 20, wherein the plant-expressible promoter sequence is selected from the list comprising the Actin1 promoter, GT1 promoter, HMW promoter, ubiquitin promoter and Bx17 promoter sequences.

22. The method according to claim 18, wherein the transcription termination sequence is the *Agrobacterium tumefaciens* nopaline synthase terminator or the rice ADP-glucose

pyrophosphorylase gene terminator.

23. The method according to claim 18, wherein the genetic construct further comprises the *shrunken*1 intron sequence placed between the promoter sequence and the genetic sequence which encodes the iron-binding protein.

24. A non-animal organism produced using the method according to any one of claims 1 to 23, wherein said non-animal organism or a cell, tissue or organ thereof comprises a higher level of bioavailable iron in a protein-bound form than an otherwise isogenic organism which does not contain the introduced genetic sequence encoding the iron-binding protein.

25. The non-animal organism according to claim 24 being a plant.

26. A plant produced using the method according to any one of claims 1 to 23, wherein the seed of said plant comprises a higher level of bioavailable iron in a protein-bound form than an otherwise isogenic plant which does not contain the introduced genetic sequence encoding the iron-binding protein.

27. Progeny of the plant according to claim 26 capable of producing seed which contain a higher level of bioavailable iron in a protein-bound form than the seed of an otherwise isogenic plant which does not contain the introduced genetic sequence encoding the iron-binding protein.

28. An isolated nucleic acid molecule which comprises a nucleotide sequence which is at least about 85% identical to the nucleotide sequence set forth in SEQ ID NO:1 or a complementary nucleotide sequence thereto when used to increase the bioavailable iron content in a plant.

29. An isolated nucleic acid molecule which comprises a nucleotide sequence which is at least about 85% identical to the nucleotide sequence set forth in SEQ ID NO:3 or a complementary nucleotide sequence thereto or a homologue, analogue or derivative of said

nucleotide sequence which is capable of encoding an iron-binding peptide, polypeptide or protein.

30. The isolated nucleic acid molecule according to claim 29 when used to increase the bioavailable iron content in a plant.

31. An isolated nucleic acid molecule which comprises a nucleotide sequence which is at least about 85% identical to the nucleotide sequence set forth in SEQ ID NO:5 or a complementary nucleotide sequence thereto when used to increase the bioavailable iron content in a plant.

32. An isolated nucleic acid molecule which comprises a nucleotide sequence which encodes a peptide, polypeptide or protein having at least about 85% amino acid sequence similarity to the sequence set forth in SEQ ID NO:2 when used to increase the bioavailable iron content in a plant.

33. An isolated nucleic acid molecule which comprises a nucleotide sequence which encodes an iron-binding peptide, polypeptide or protein having at least about 85% amino acid sequence similarity to the sequence set forth in SEQ ID NO:4.

34. The isolated nucleic acid molecule according to claim 33 when used to increase the bioavailable iron content in a plant.

35. An isolated nucleic acid molecule which comprises a nucleotide sequence which encodes a peptide, polypeptide or protein having at least about 85% amino acid sequence similarity to the sequence set forth in SEQ ID NO:6 when used to increase the bioavailable iron content in a plant.

36. A cell which has been transformed or transfected with the isolated nucleic acid molecule according to any one of claims 28 to 35.

37. The cell according to claim 37 being a plant cell.

38. Plant tissue other than a whole plant regenerated from the cell according to claim 37.

39. The cell according to claim 37 being a bacterial cell.

40. The non-animal organism according to claims 24 or 25 when used for the preparation of a medicament or medicated foodstuff for the treatment of iron deficiency in an animal or human subject.

41. The plant according to claims 26 or 27 when used for the preparation of a medicament or medicated foodstuff for the treatment of iron deficiency in an animal or human subject.

42. The cell according to claims 36 or 37 when used for the preparation of a medicament or medicated foodstuff for the treatment of iron deficiency in an animal or human subject.

43. The plant tissue according to claim 38 when used for the preparation of a medicament or medicated foodstuff for the treatment of iron deficiency in an animal or human subject.

44. A method of treatment of iron deficiency in a human or animal subject comprising administering to said subject plant tissue or a derivative thereof having a high bioavailable iron content for a time and under conditions sufficient for the level of iron detectable in the blood of said subject to increase, wherein said plant tissue has a high bioavailable iron content by virtue of the expression therein of an introduced genetic sequence which encodes and iron-binding peptide, polypeptide or protein.

45. The method according to claim 44, wherein the introduced genetic sequence comprises the isolated nucleic acid molecule according to any one of claims 28 to 35.

46. The method according to claims 44 or 45, wherein the plant tissue comprises an edible

plant or plant part.

47. The method according to claim 46, wherein the edible plant part is seed.

48. The method according to claim 47 wherein the seed is rice seed.

49. The method according to claim 44, wherein the plant tissue or a derivative thereof is a medicated foodstuff and administered orally.

50. The method according to claim 44, wherein the derivative of the plant tissue comprises a liquid tonic comprising the iron-binding protein or iron and said tonic is administered orally.

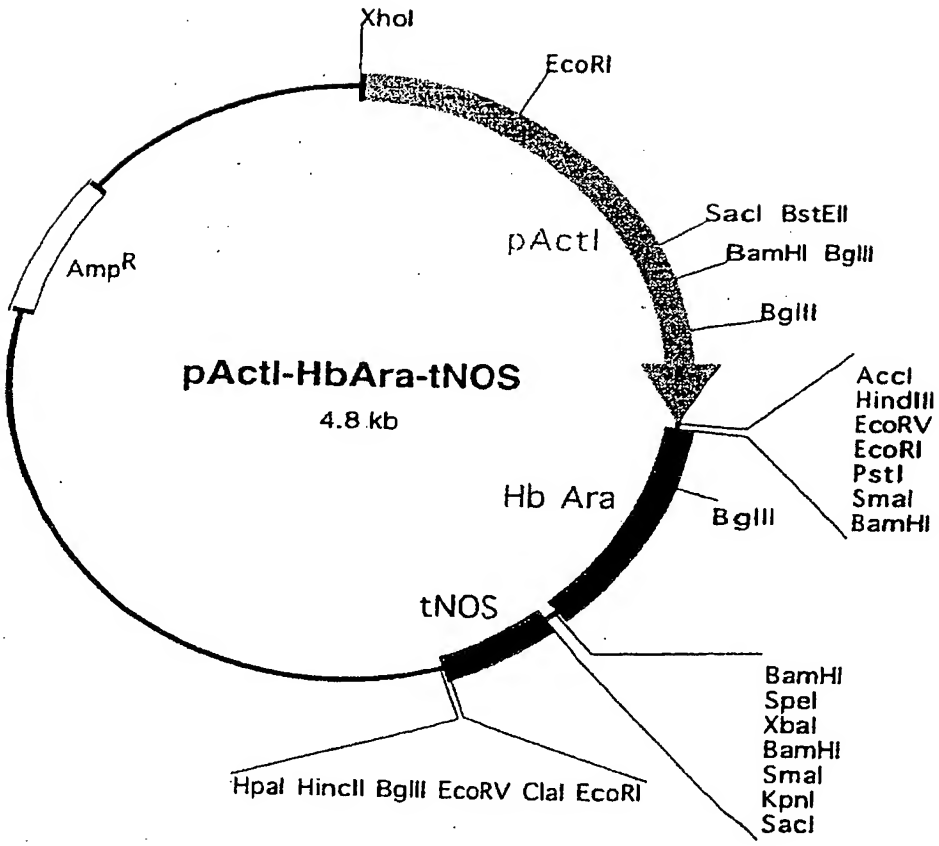


FIGURE 1

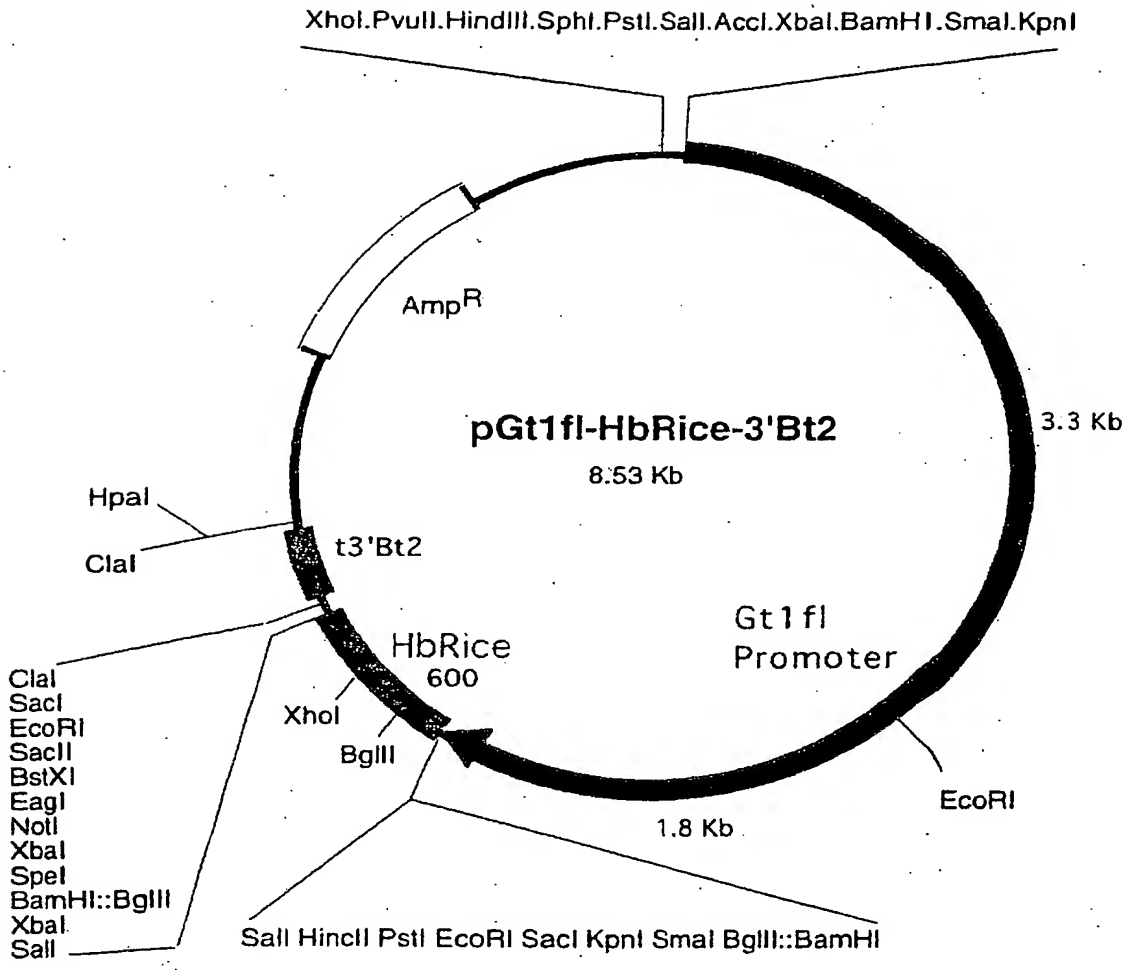
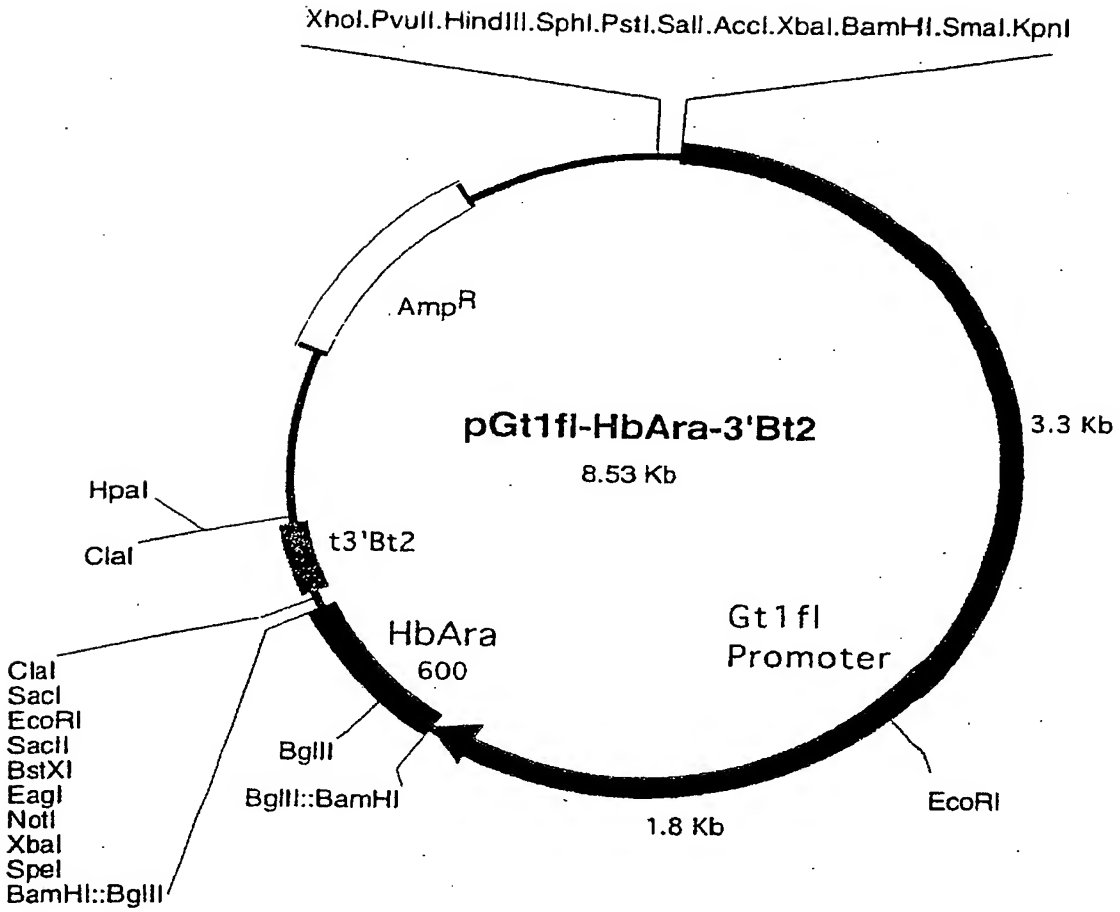


FIGURE 2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AmpR
pGt1fl-HbAra-3'Bt2
8.53 Kb
3.3 Kb
HpaI
ClaI
t3'Bt2
HbAra
600
Gt1fl
Promoter
ClaI
SacI
EcoRI
SacII
BstXI
EagI
NotI
XbaI
SpeI
BamHI::BglII
BglII
BglII::BamHI
1.8 Kb
EcoRI

FIGURE 3

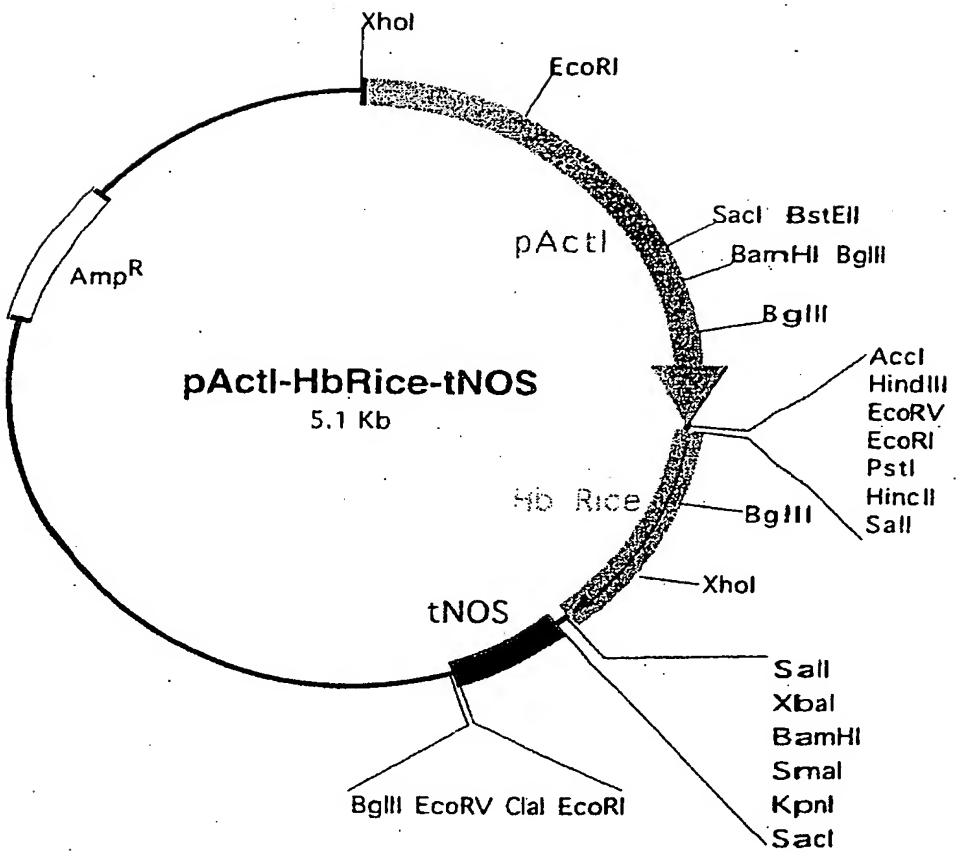


FIGURE 4

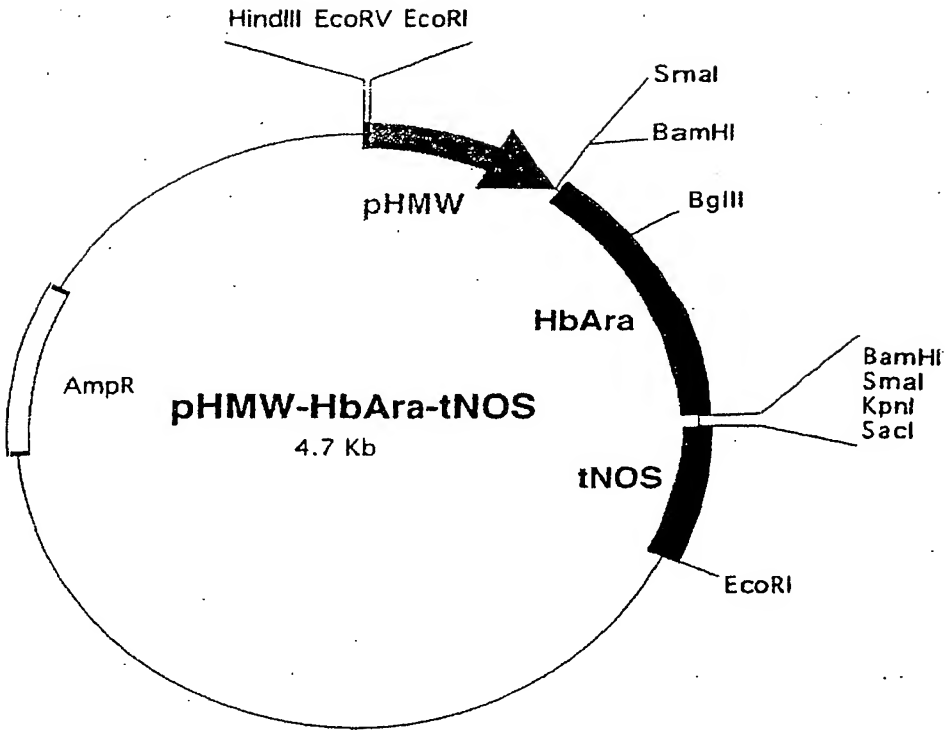
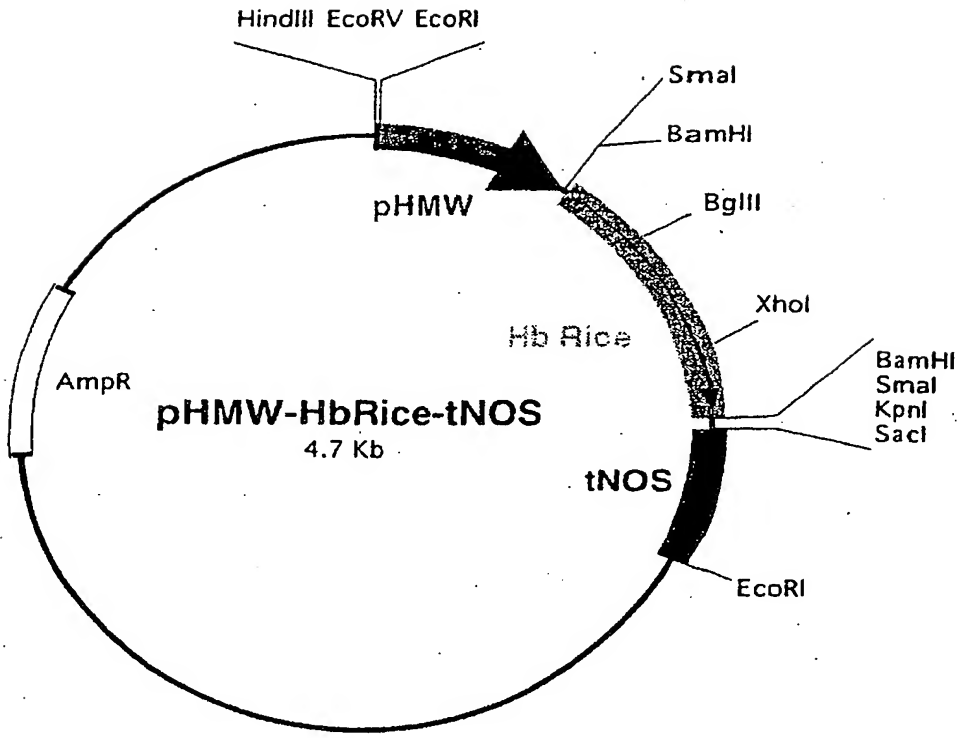


FIGURE 5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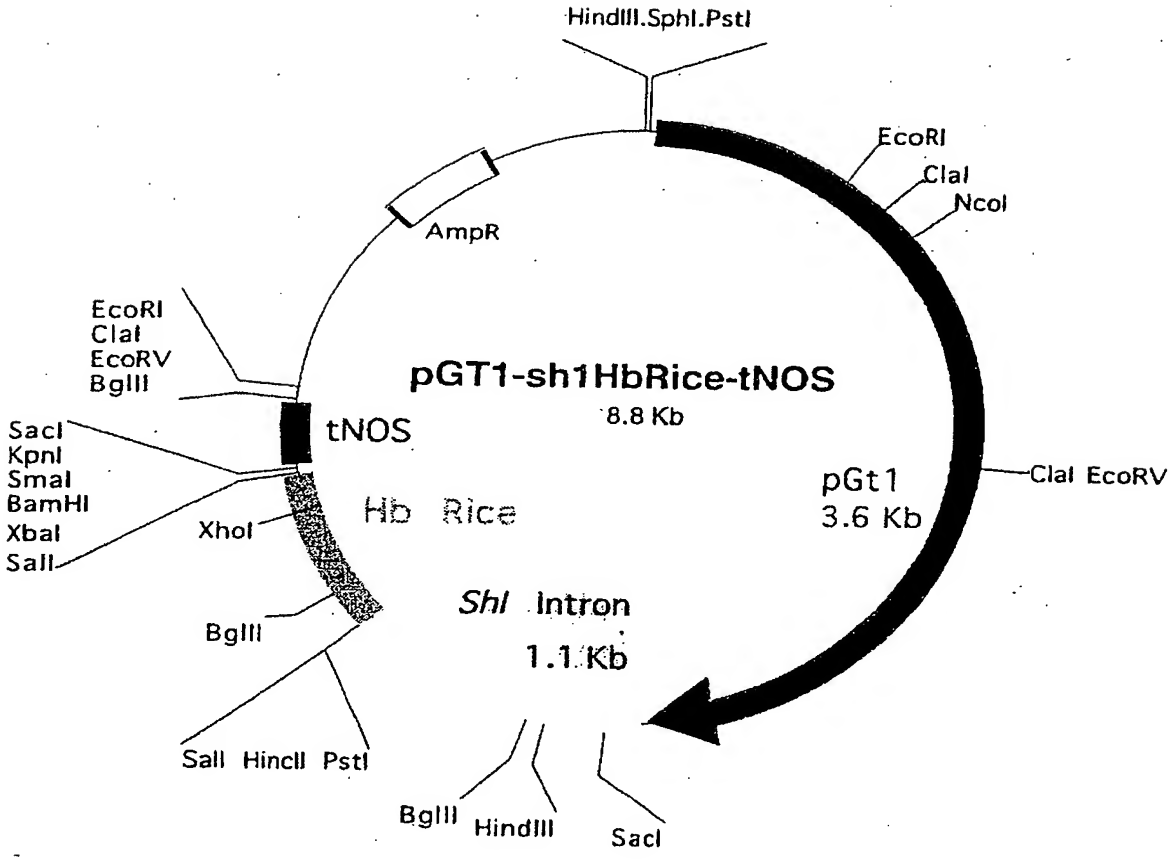
SmaI
BamHI
pHMW
BglII
XhoI
Hb Rice
BamHI
SmaI
KpnI
SacI
AmpR
pHMW-HbRice-tNOS
4.7 Kb
tNOS
EcoRI

FIGURE 6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ClaI
NcoI
AmpR
EcoRI
ClaI
EcoRV
BglII
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8.8 Kb
tNOS
SacI
KpnI
SmaI
BamHI
XbaI
SalI
Hb Rice
XhoI
pGt1
3.6 Kb
ClaI EcoRV
Shl Intron
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BglII
SalI HincII PstI
BglII
HindIII
SacI

FIGURE 7

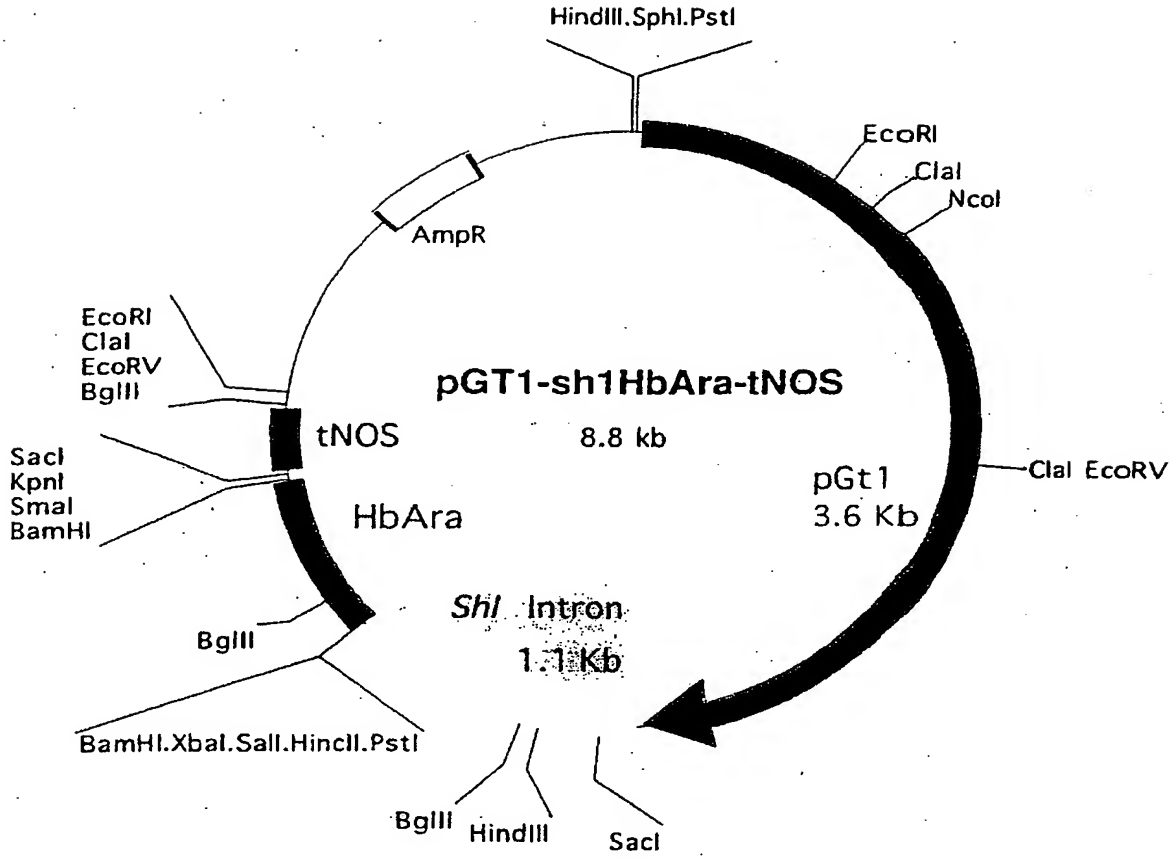


FIGURE 8

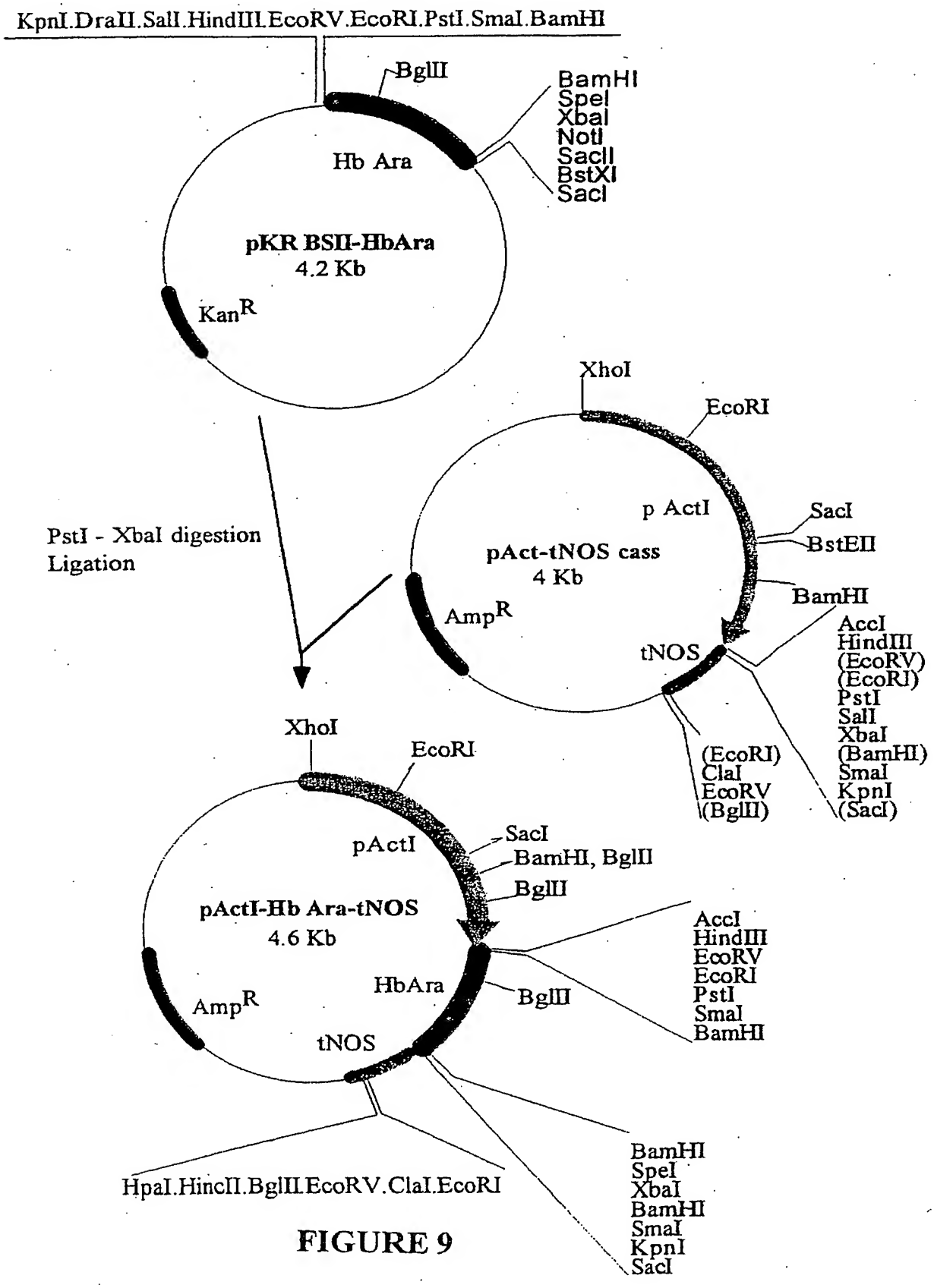


FIGURE 9

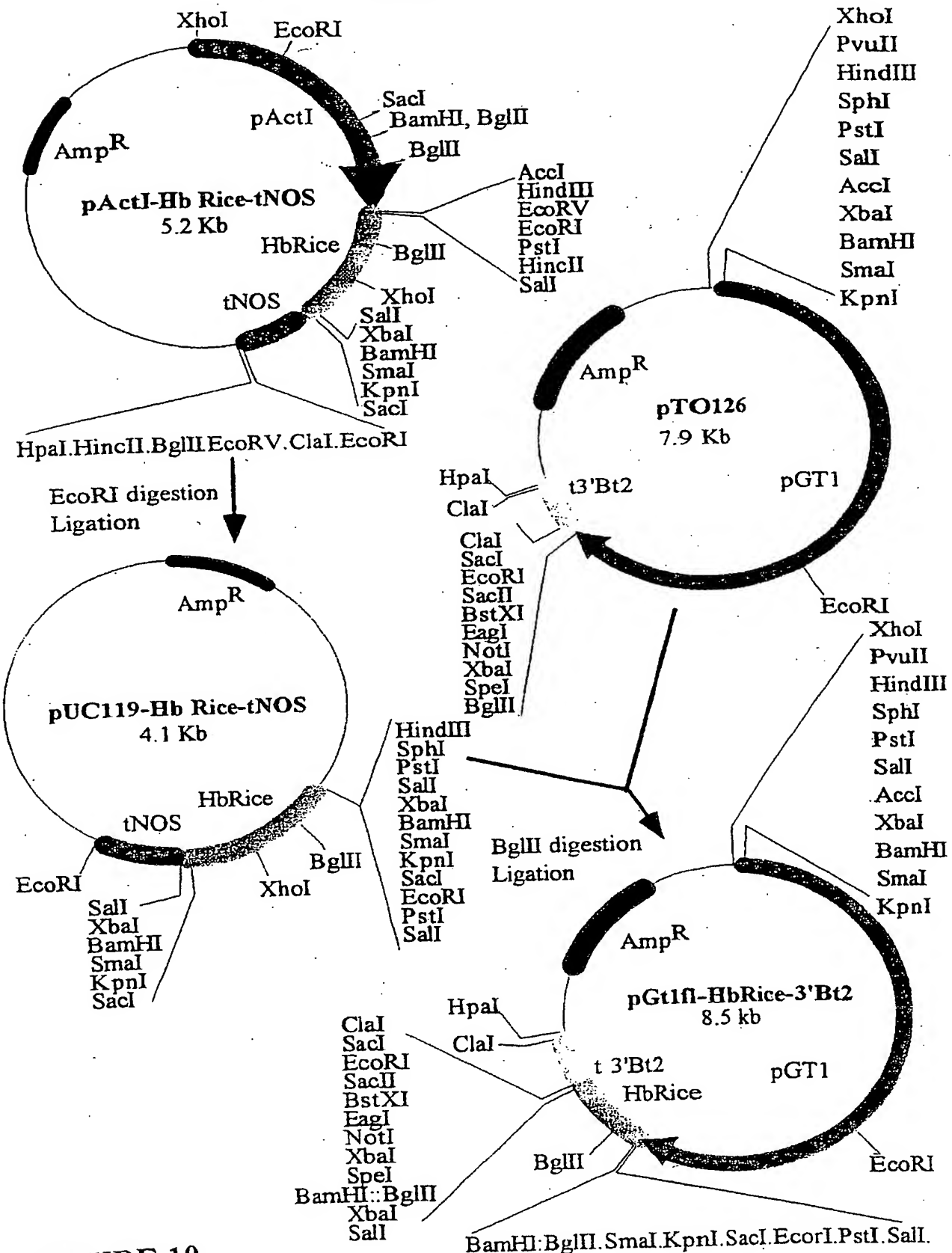


FIGURE 10

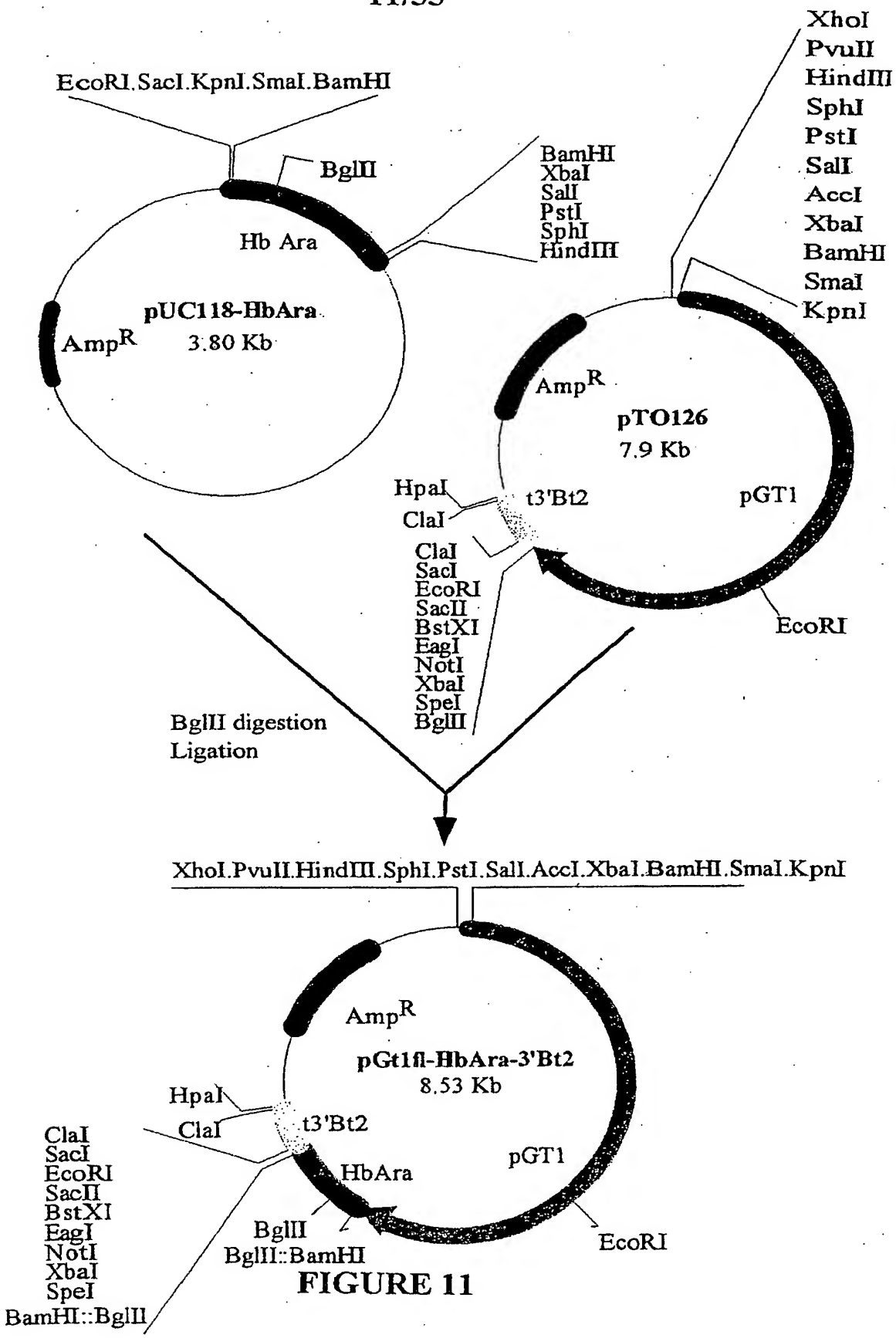


FIGURE 11

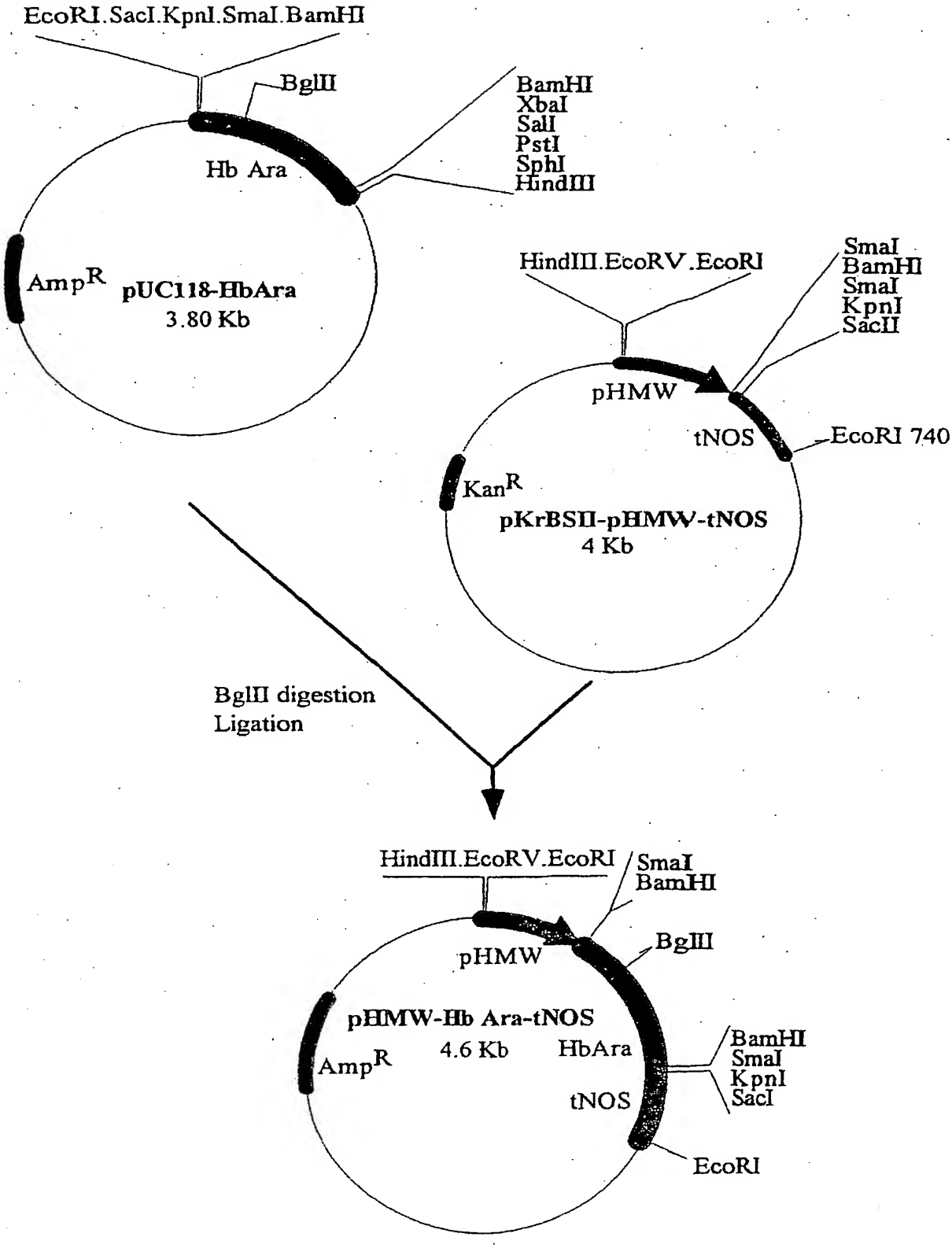


FIGURE 12

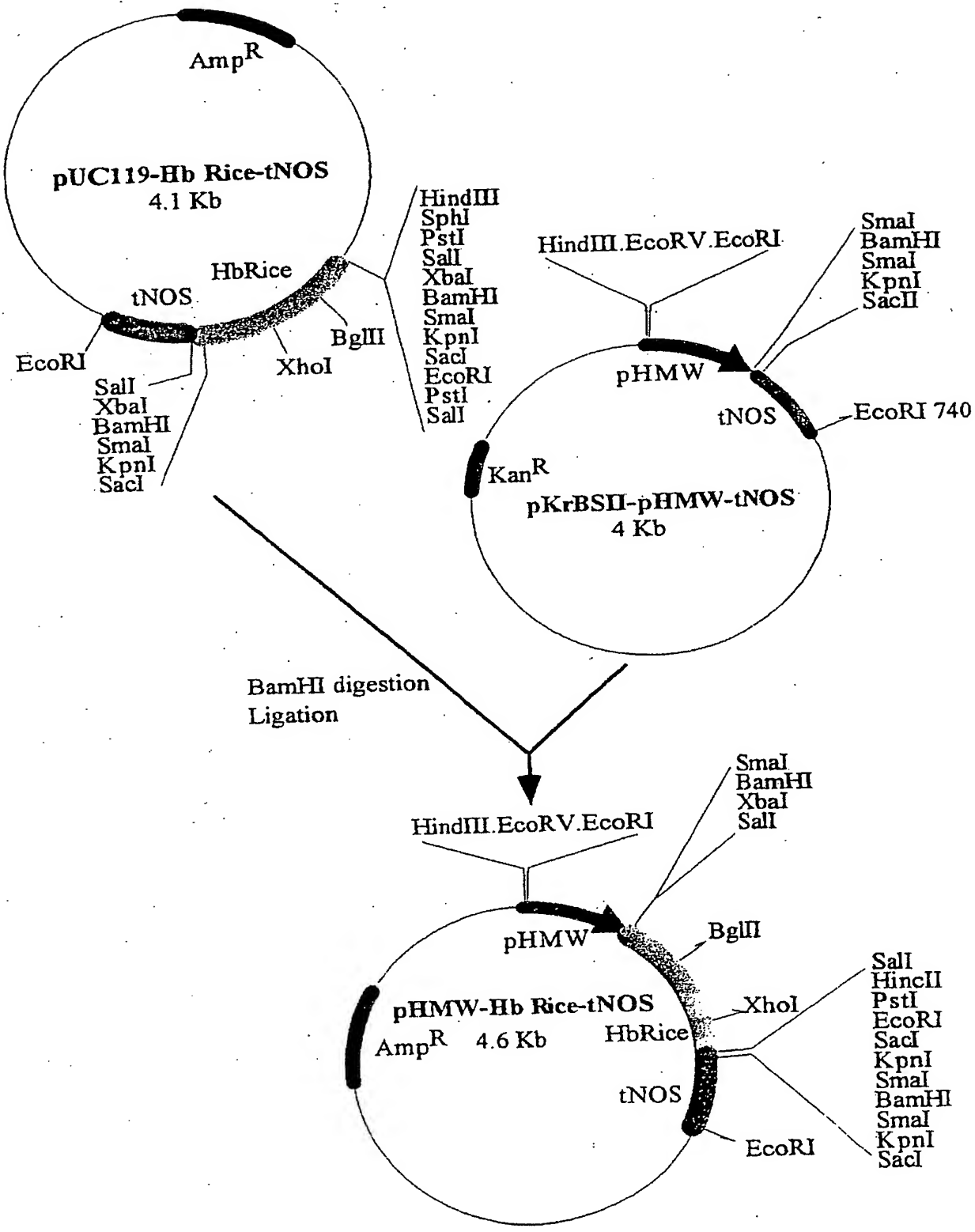


FIGURE 13

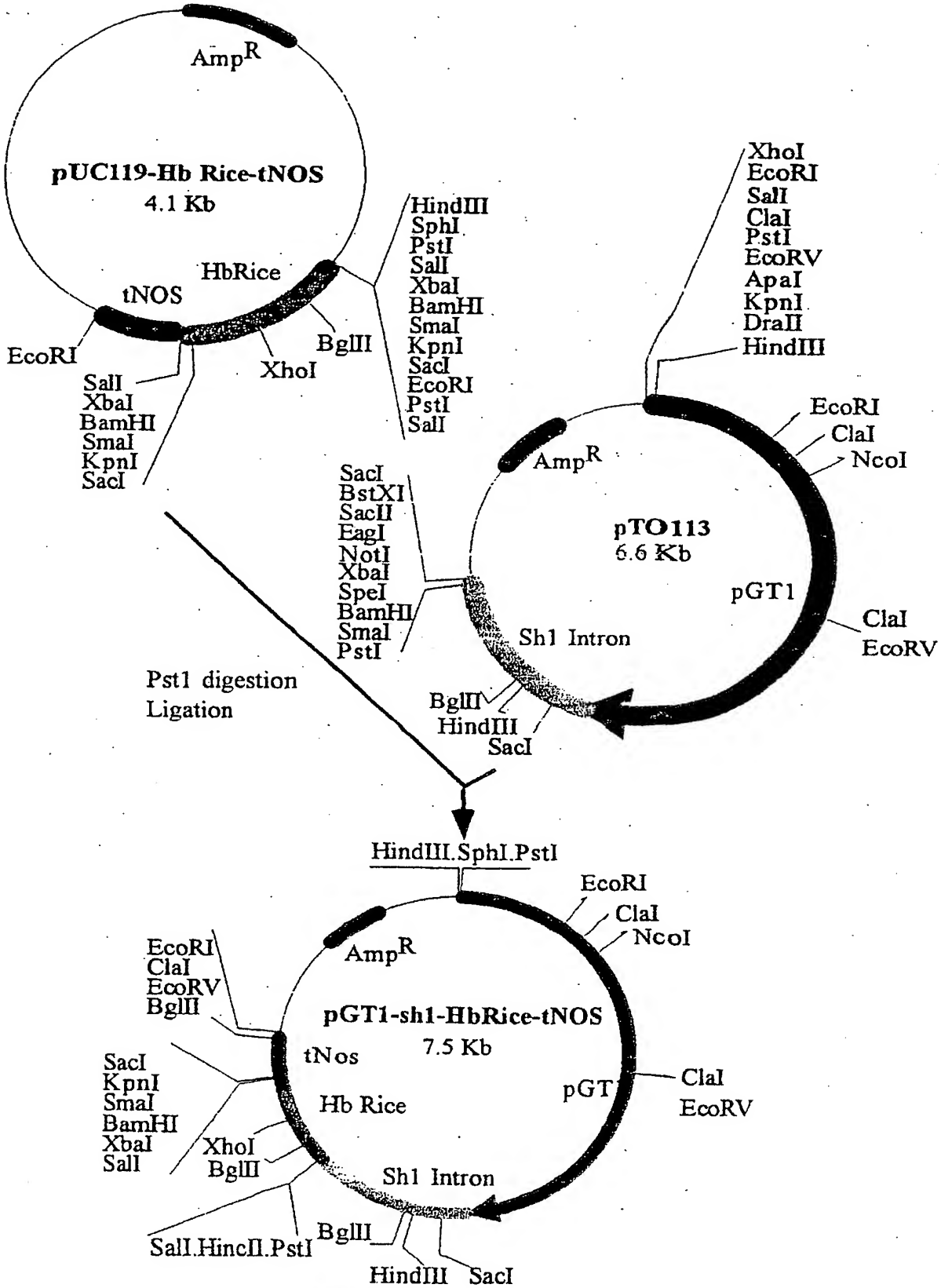


FIGURE 14

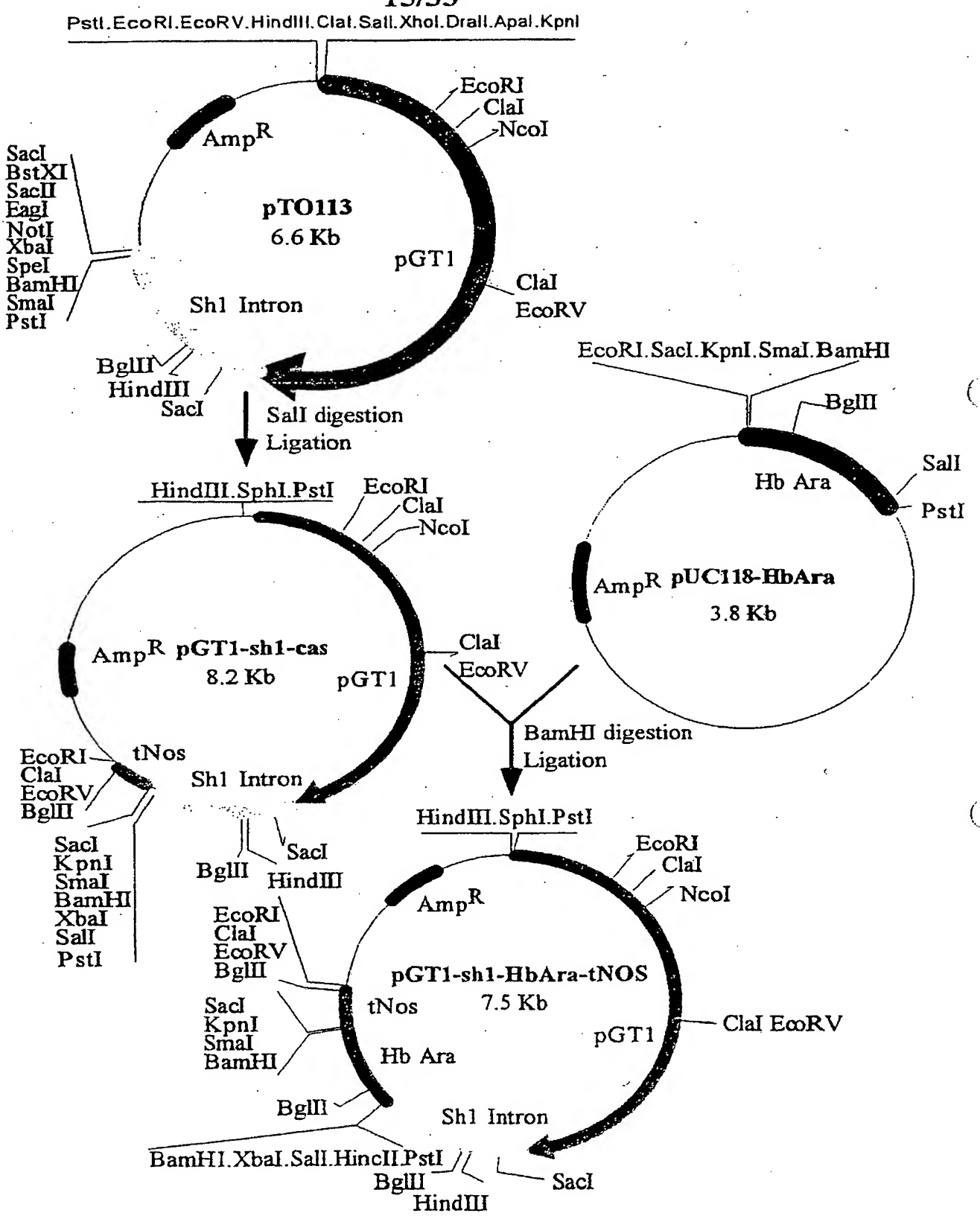


FIGURE 15

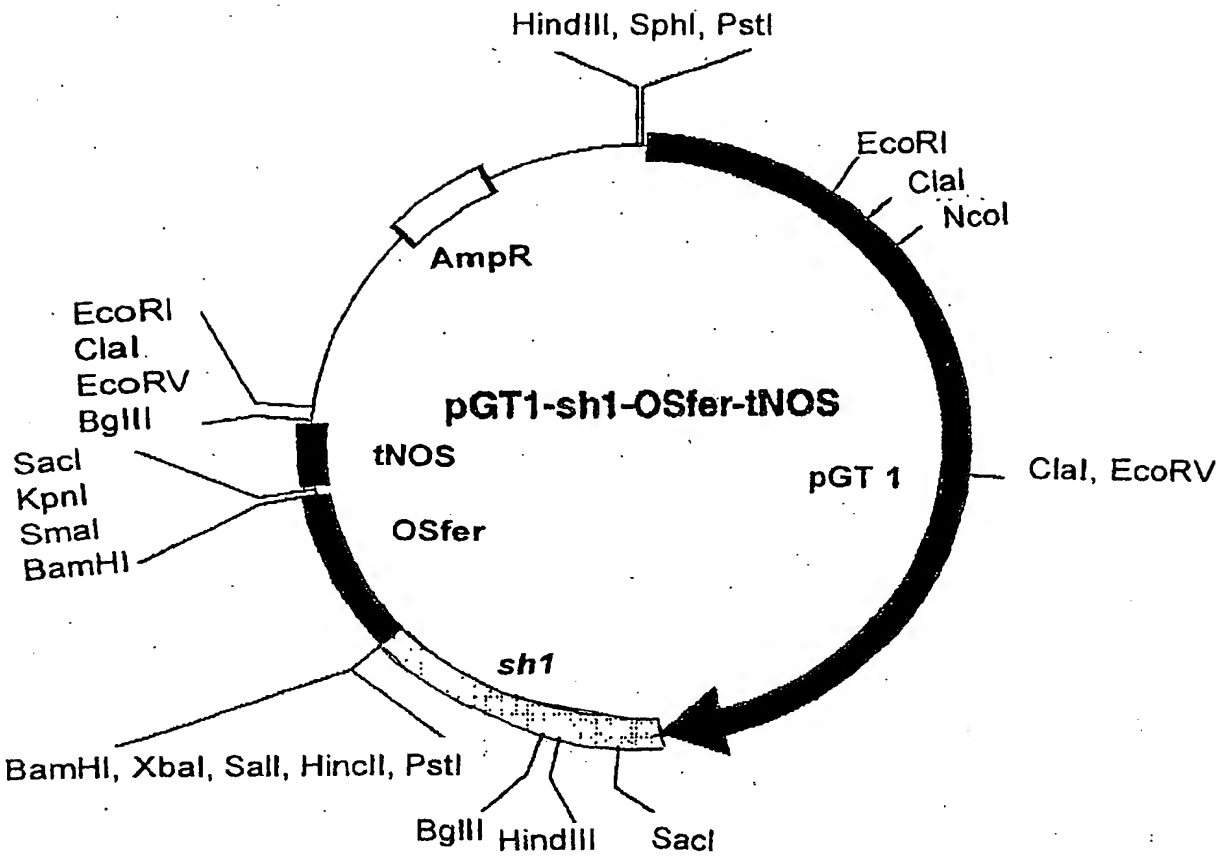


FIGURE 16

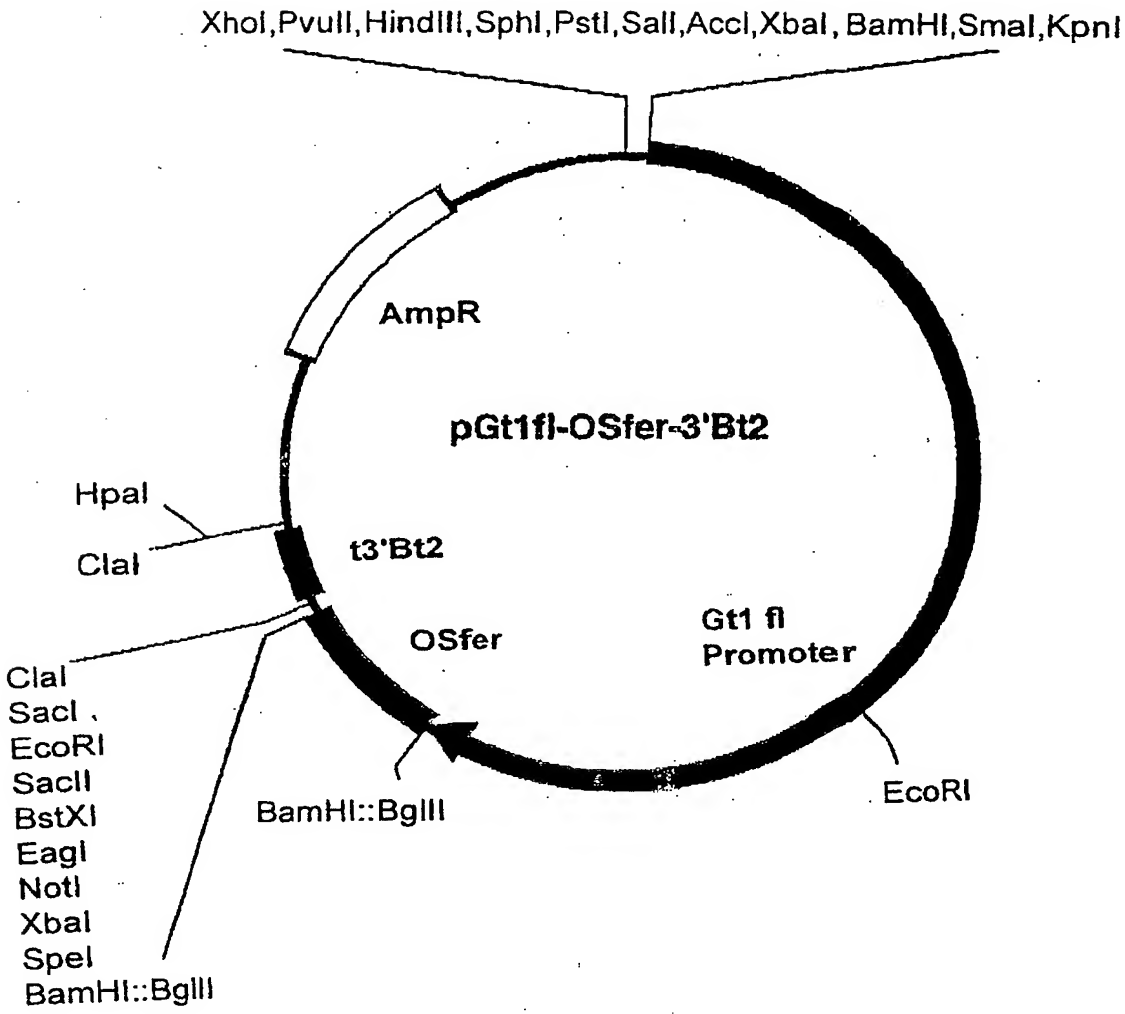


FIGURE 17

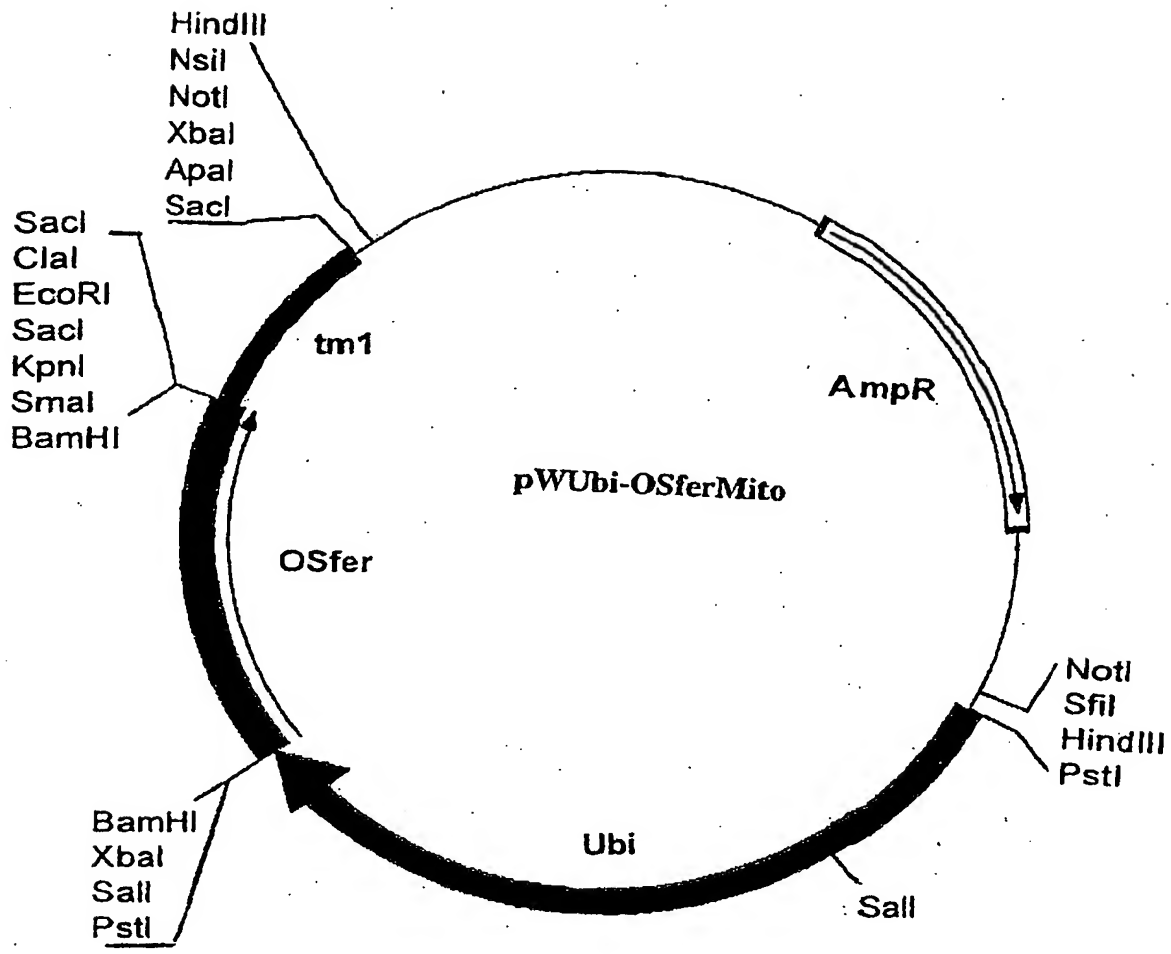


FIGURE 18

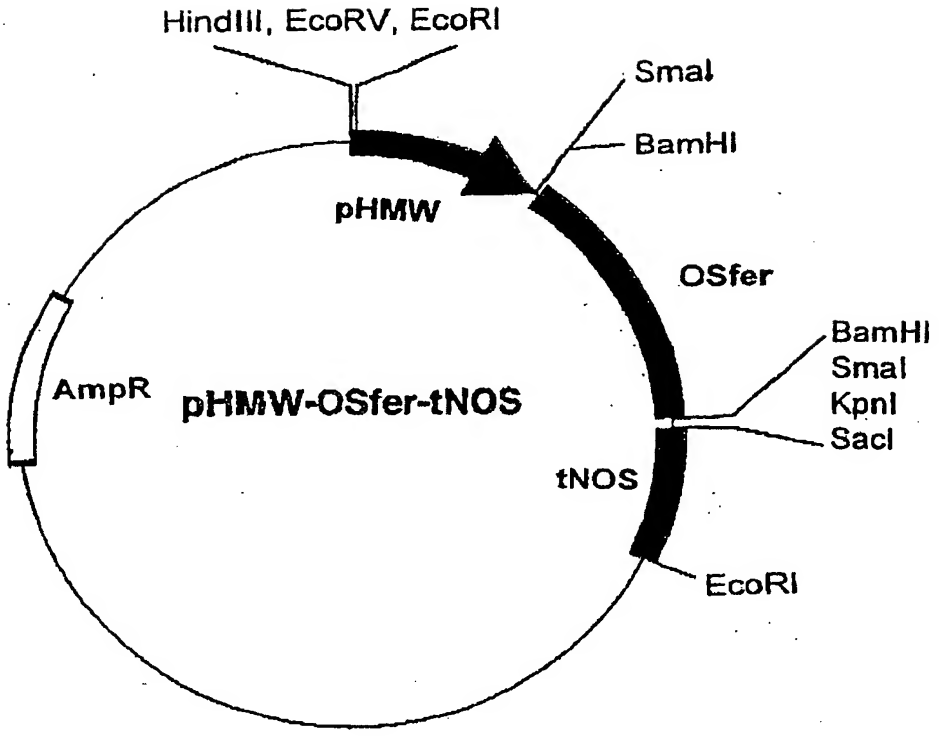


FIGURE 19

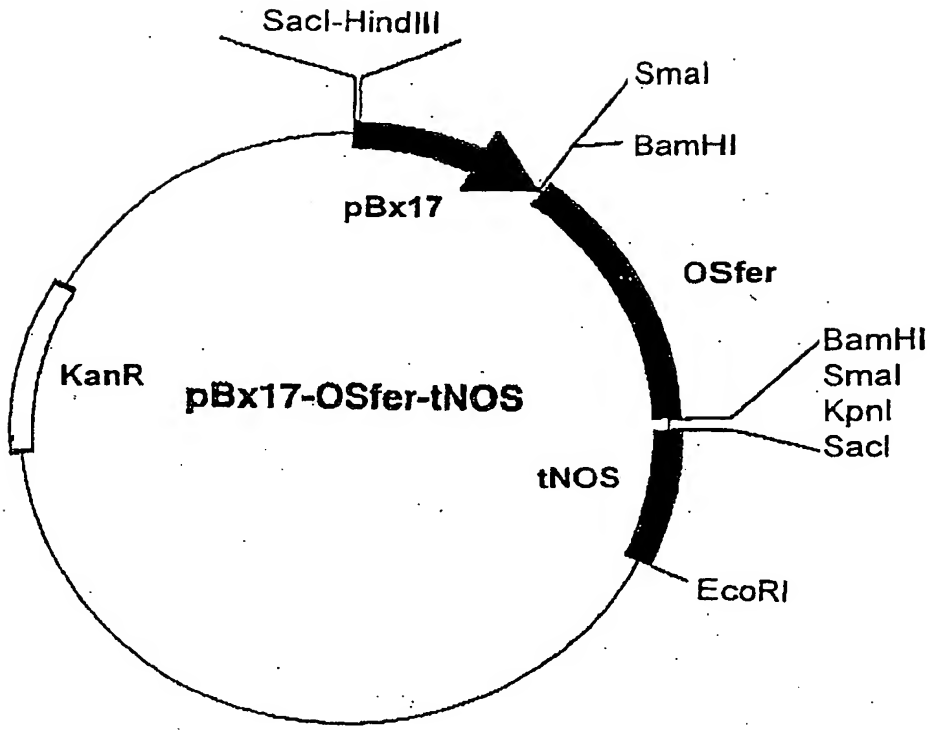
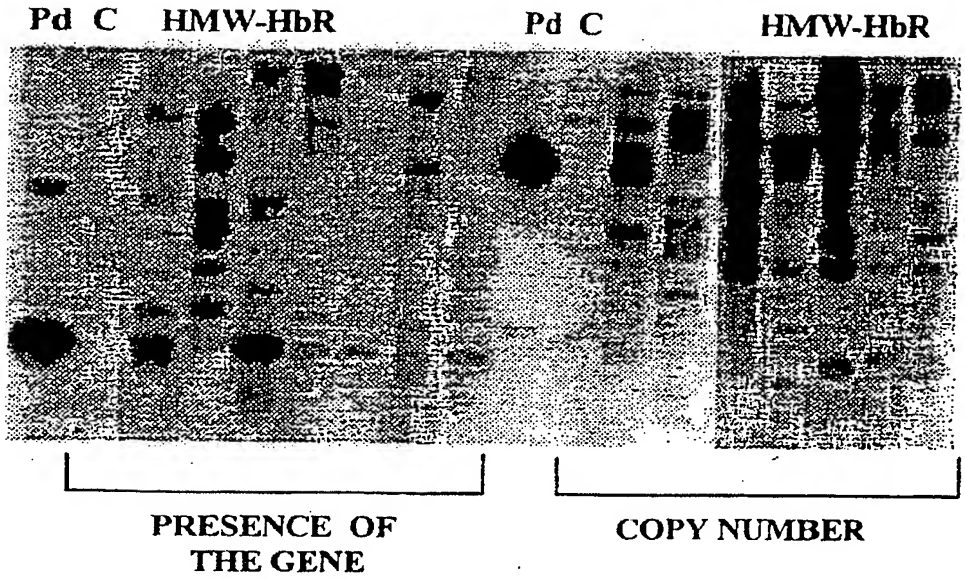


FIGURE 20



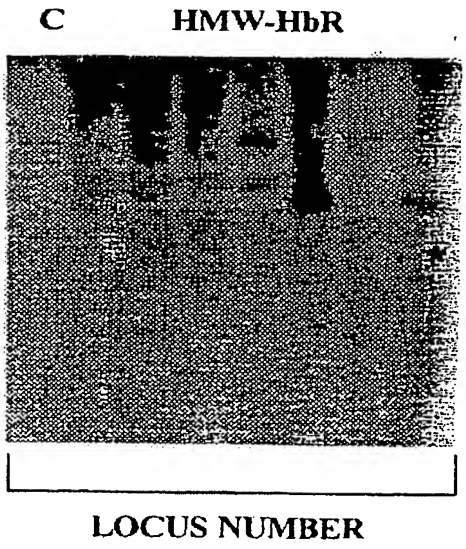


FIGURE 21

C INDIVIDUAL T1 PLANTS HMW - HbR

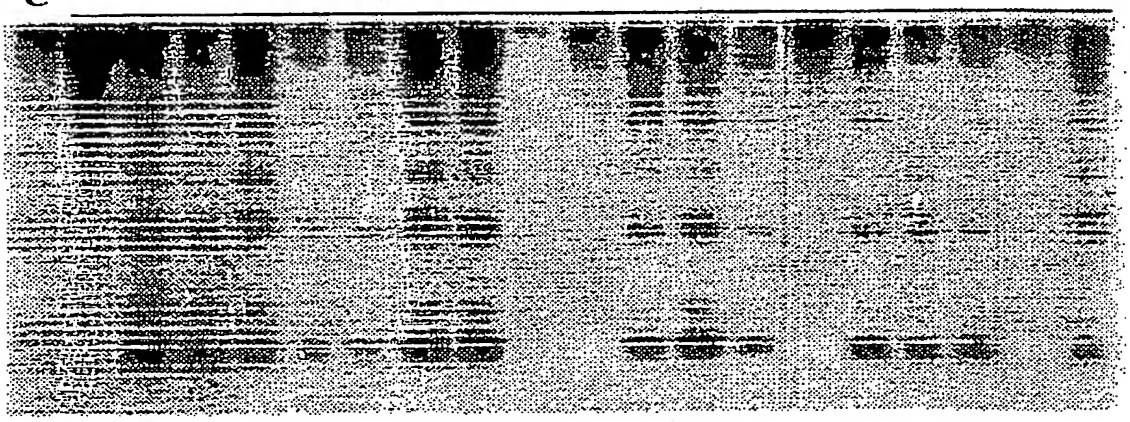


FIGURE 22

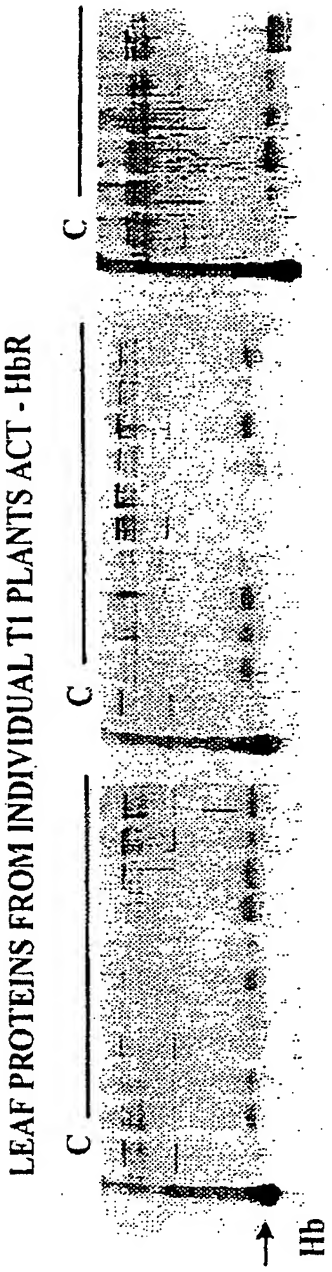


FIGURE 23

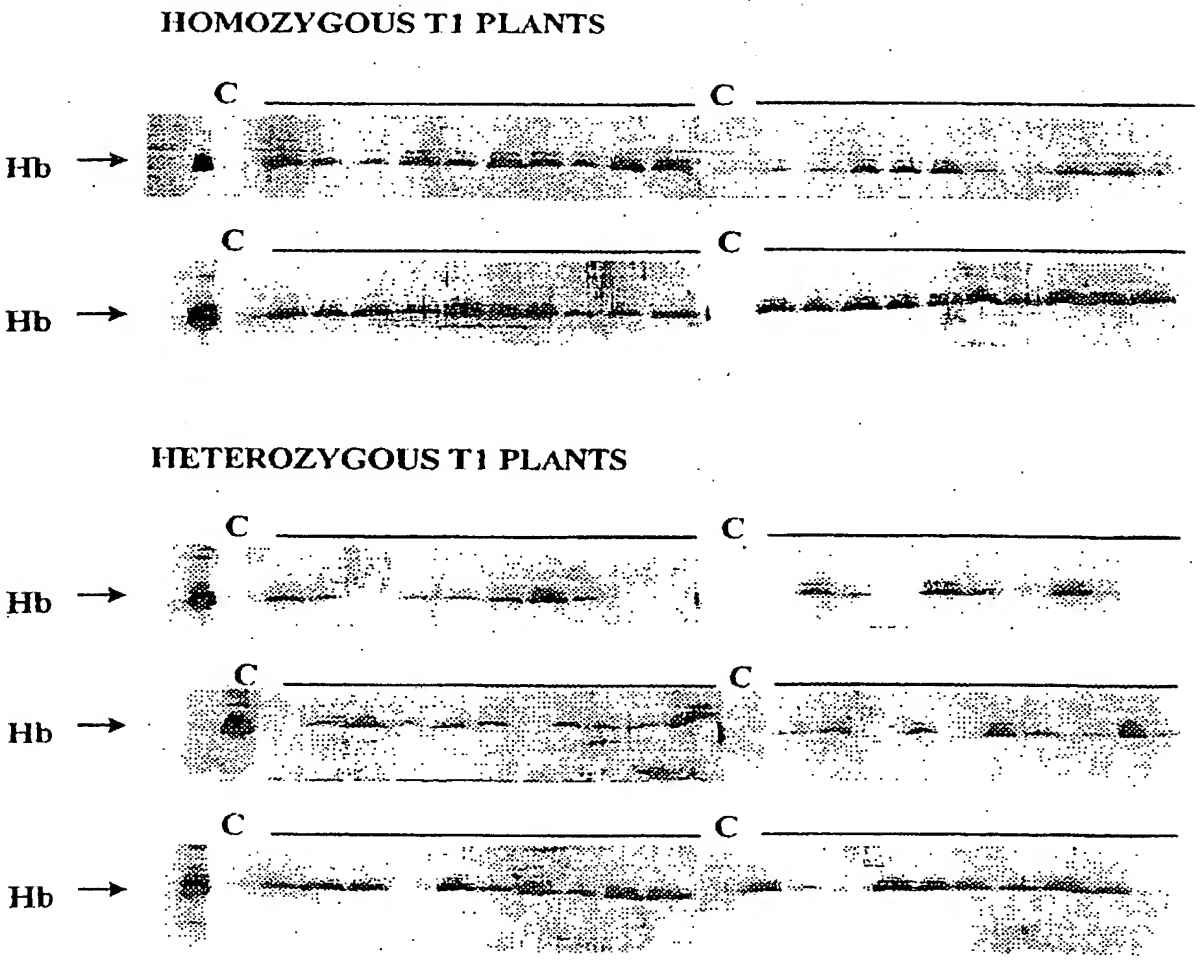


FIGURE 24

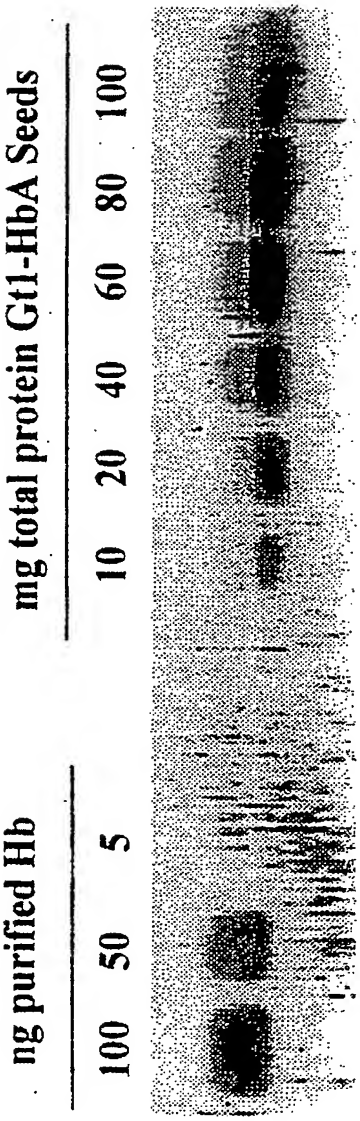
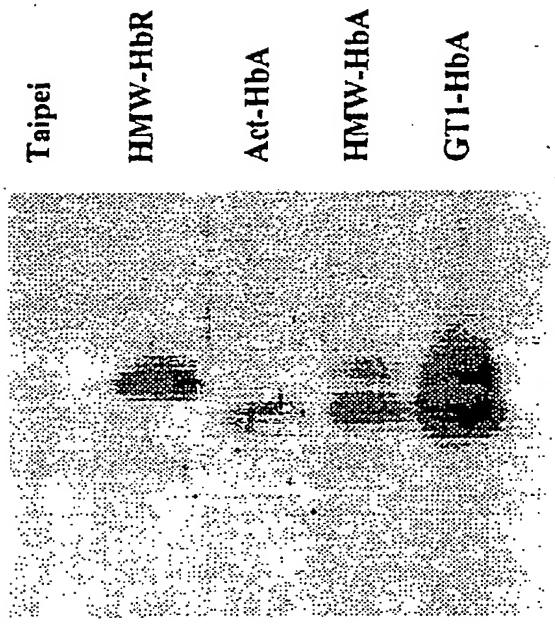


FIGURE 25



Seed protein extracts
from homozygous T1 plants

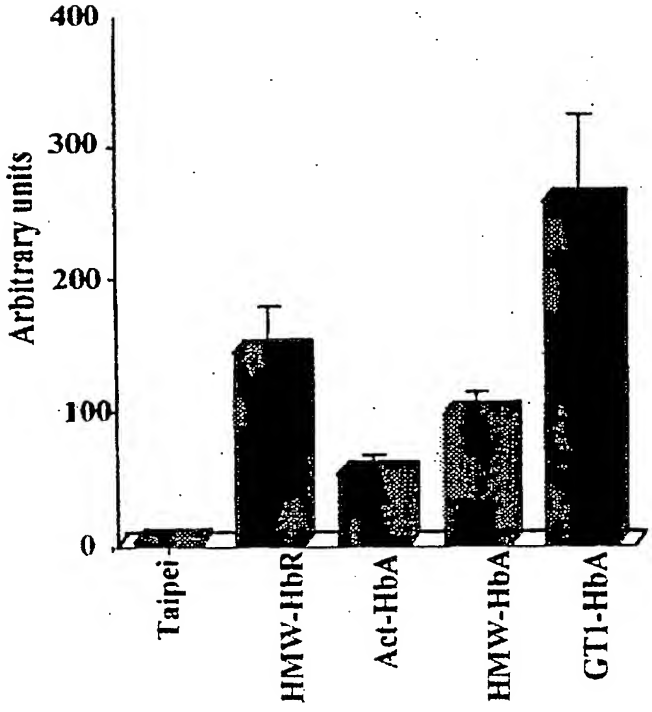


FIGURE 26

```
OSFer   1 MMLPPRVAPAAAAAAPTYLAAAASTPAS.VWLPVPRG..AGPGAVCRAA 46
          |||  ||.|.:|||.||  |.:|:.|||.  |:::.|||    :::||.||||
FM1     1 MML..RVSPSPAAAVPTQLSGAPATPAPVVRVAAPRGVASPSAGAACRAA 48

OSFer  47 GKGKEVLSGVVFQPFEELKGELSLVPQAKDQSLARQKFVDECEAAINEQI 96
          ||||||||||||||||||:||||.||||..|.||||:||||:|||||:|||||
FM1    49 GKGKEVLSGVVFQPFEEIKGELALVPQSPDKSLARHKFVDDCEAALNEQI 98

OSFer  97 NVEYNASYAYHSLFAYFDRDNVALKGFAKFFKESSDEERDHAEKLIKYQN 146
          |||||||||||||||||||||||||||||||||||||:||||||:.|||
FM1    99 NVEYNASYAYHSLFAYFDRDNVALKGFAKFFKESSDEEREHAEKLMEYQN 148
```

FIGURE 27-1

```
OSFer  147 MRGGRVRLQSIVTPLTEFDHXEKGDALYAMELALALEKLVNEKLHNLHSV 196
           |||||||||||||||||||| |||||||||||||||||||||||||:|
FM1    149 KRGGRVRLQSIVTPLTEFDHPEKGDALYAMELALALEKLVNEKLHNLHGV 198

OSFer  197 ASRCNDPQLTDFVESEFLEEQVEAIKKISEYVAQLRRVGKGHGVWHFDQK 246
           |.||||||||||:||||||||||.|||.|||.||||||||||||||||||.
FM1    199 ATRCNDPQLTDFIESEFLEEQGEAINKISKYVAQLRRVGKGHGVWHFDQM 248

OSFer  247 LLEEEA*
           |||||||.:.
FM1    249 LLEEEA*
```

FIGURE 27-2

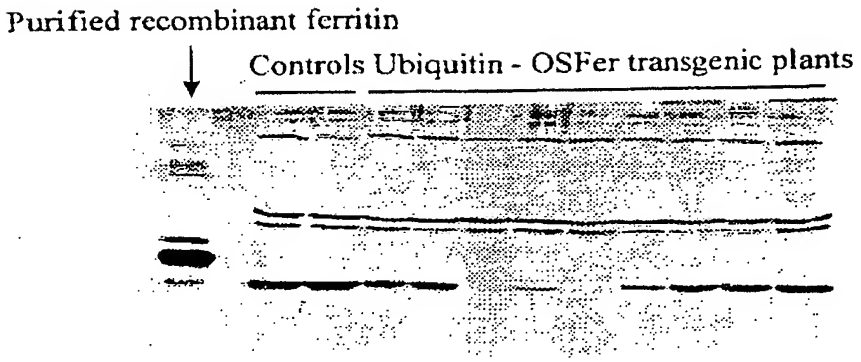


FIGURE 28

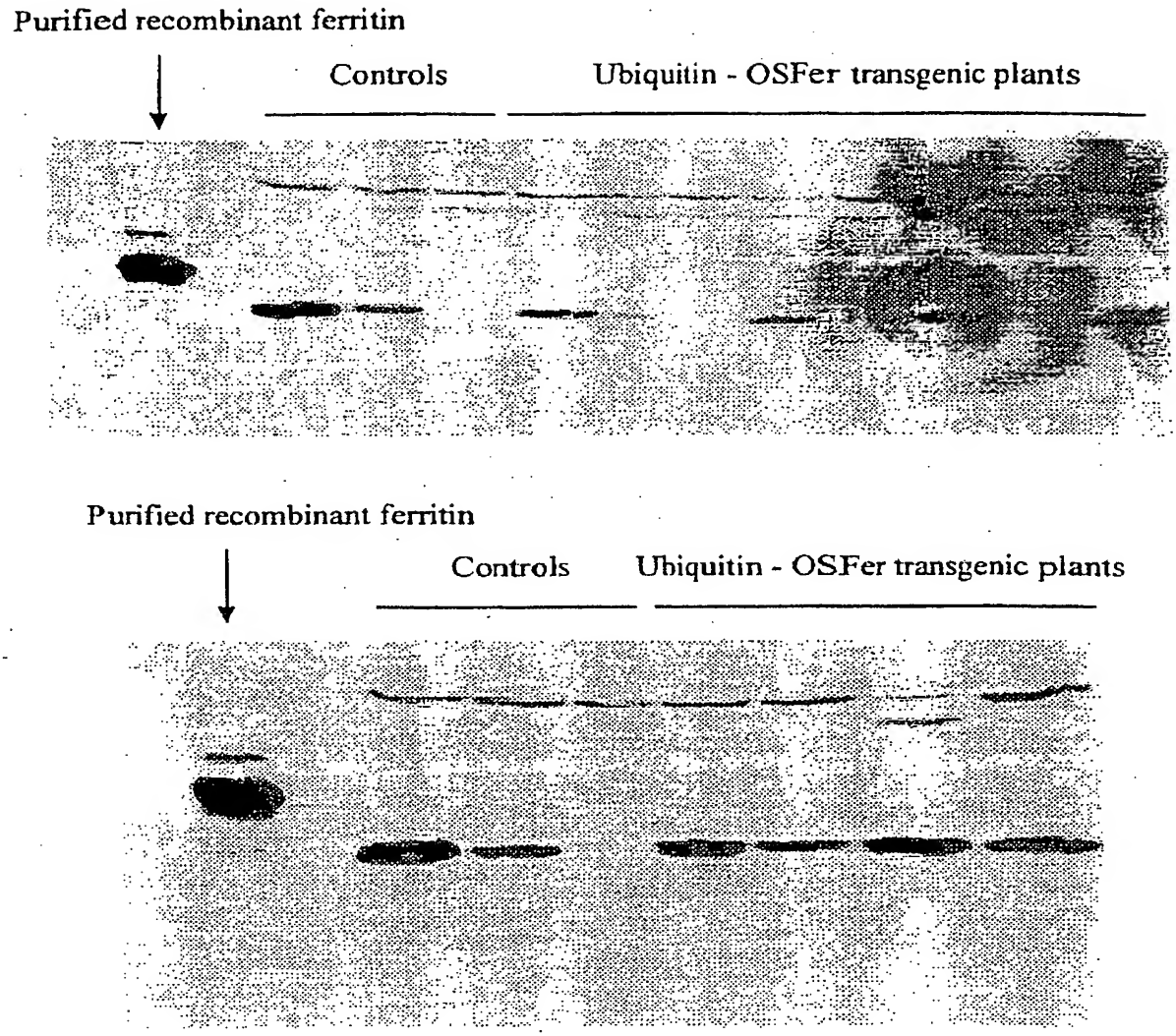


FIGURE 29

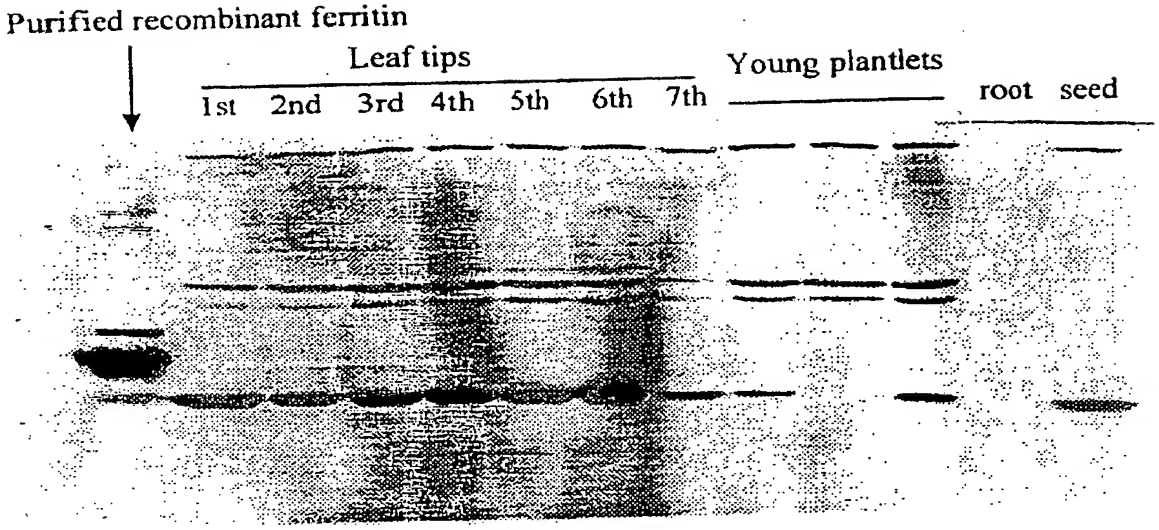


FIGURE 30

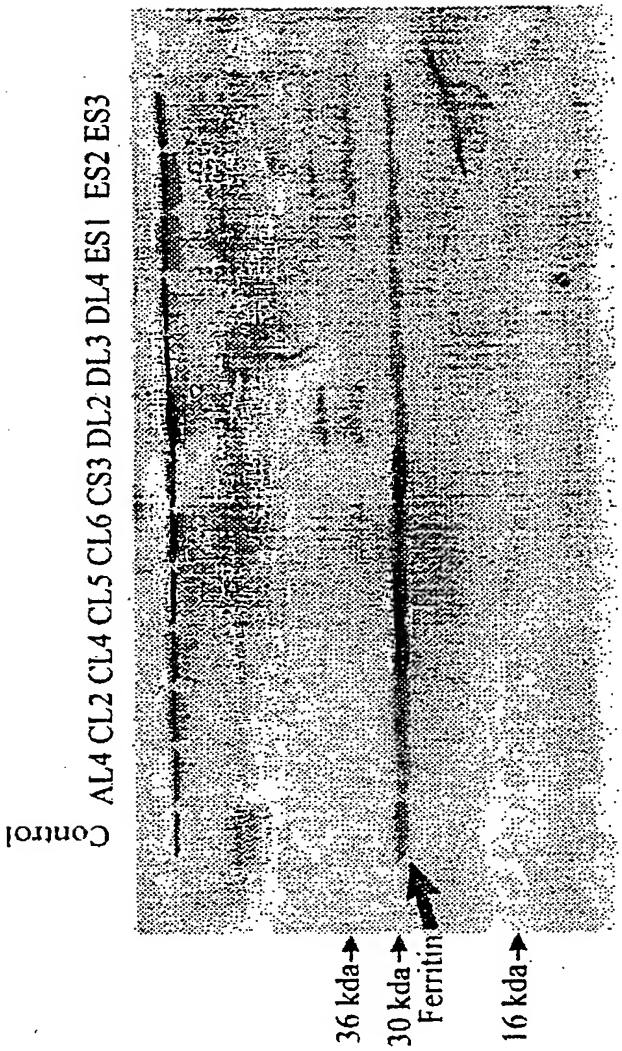


FIGURE 31

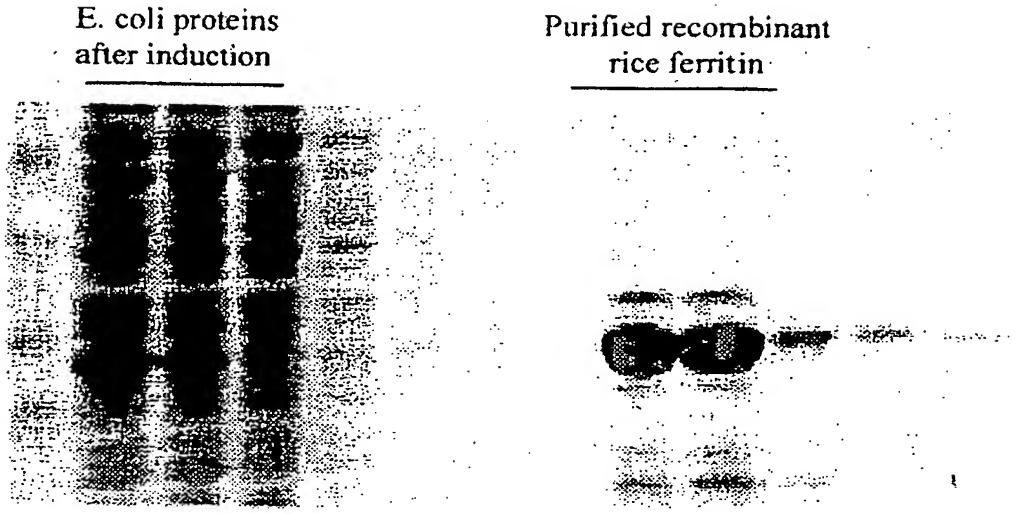


FIGURE 32

INTERNATIONAL SEARCH REPORT

International Application No.
PCT/AU 98/00526

A. CLASSIFICATION OF SUBJECT MATTER

Int Cl[6]: C12N 15/29, A01H 5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N, A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
CHEMICAL ABSTRACTS

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
WPAT, JAPIO (DERWENT); AGRICOLA (CHEMICAL ABSTRACTS); GENBANK; EMBL; SWISSPROT (SEQUENCE SEARCH); KEYWORDS (IRON, HEME, FERRETIN)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, X	PLANT PHYSIOLOGY, vol.115, 1259-1266; Arredondo-Peter, R. et al. (1997); Rice Hemoglobins. See whole document	1-36, 40-41 44-50
P, X	TRANSGENIC RESEARCH, vol.7, 173-180; Goto, F. et al. (1998); Iron accumulation in tobacco plants expressing soyabean ferritin gene; See whole document	1-36, 40-41 44-50
P, X	JP 09-201190 A and Chemical Abstracts; abstract no. 127: 215964 See abstract	1-36, 40-41 44-50

[X] Further documents are listed in the continuation of Box C

[X] See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" Earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
9 September 1998	15 SEP 1998
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200 WODEN ACT 2606 AUSTRALIA Facsimile No.: (02) 6285 3929	Authorized officer **David Hennessy** Telephone No.: (02) 6283 2255

Form PCT/ISA/210 (second sheet) (July 1992) copdmi

INTERNATIONAL SEARCH REPORT

International Application No.
PCT/AU 98/00526

C (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, X	WO 98/12913 A1 (Bailey and Bulow) 2 April 1998 See the examples	1-36, 40-41 44-50
X	WO 92/16634 (Novo Nordisk A/S) 1 October 1992 See the examples	1-6, 18 20-27, 40-41
X	WO 93/19195 (Novo Nordisk A/S) 30 September 1993 See the examples	1-6, 18 20-27, 40-41
X	WO 93/25697 (California Institute of Technology) 23 December 1993 See the examples	1-36, 40-41
X, Y	PLANT MOLECULAR BIOLOGY, vol.24, 853-862; Taylor, E.R. et al. (1994); A cereal haemoglobin gene is expressed in seed and root tissues under anaerobic conditions. See whole article	1-6, 9-14 16-28, 32, 36 40-41
X, Y	PLANT MOLECULAR BIOLOGY, vol.19, 563-575; Lobreaux, S. et al. (1992); Iron induces ferritin synthesis in maize plantlets. See whole article	1-6, 9-14 16-28, 32, 36 40-41, 44-50
X, Y	MOLECULAR & GENERAL GENETICS, vol.214, 158-161; Khosla, C. and Bailey, J.E. (1988); The Vitreoscilla hemoglobin gene: Molecular cloning, nucleotide sequence and genetic expression in Escherichia coli. See whole article	1-8, 15, 19-28, 32, 36 40-41
X, Y	THE JOURNAL OF BIOLOGICAL CHEMISTRY, vol.265, no.30, 18339-18344; Ragland, M. et al. (1990); Evidence for conservation of ferritin sequences among plants and animals and for a transit peptide in soybean. See whole article	1-6, 9-14 16-27, 29-31 33-35
X, Y	MOLECULAR & GENERAL GENETICS, vol.214, 68-73; Landsmann J. et al. (1988); Organ regulated expression of the parasponia andersonii haemoglobin gene in transgenic tobacco plants. See whole article	1-8, 15, 18-28 32, 36 40-41
X, Y	BIOCHEM. J., vol.305, 253-261; Van Waytswinkel, O. et al. (1995); Purification and characterisation of recombinant pea-seed ferritins expressed in Eschericia coli: influence of N-terminus deletions on protein solubility and core formation in vitro.	1-6, 9-14 16-27, 29-31 33-36, 40-41
X, Y	NATURE BIOTECHNOLOGY, vol.15, 244-247; Holmberg, N. et al. (1997); Transgenic tobacco expressing vitreoscilla hemoglobin exhibits enhanced growth and altered metabolite production. See the discussion	1-8, 15, 18-28 32, 36, 40-41 44-50
X, Y	NATURE, vol.331, 178-180; Bogusz, D. et al. (1988); Functioning haemoglobin genes in non-nodulating plants. See the whole article	24-28, 32 40/41

INTERNATIONAL SEARCH REPORT

International Application No.
PCT/AU 98/00526

Box 1 Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 37-39, 42-43
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

Claim 37 is self-appended and this renders the claim and appended claims unclear to the extent no meaningful search could be made on these claims.

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a)

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT
Information on patent family members

International Application No.
PCT/AU 98/00526

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member					
WO	98/12913	AU	45022/97				
WO	92/16634	BR	9205802	CA	2106485	EP	505311
		FI	934135	NZ	242074	US	5744323
WO	93/19195	EP	631631	US	5681725		

END OF ANNEX